Rôle du récepteur Sigma-1 sur la régulation des canaux ioniques impliqués dans la carcinogenèse

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Résumé :

Le récepteur sigma-1 est une protéine chaperonne active dans des tissus lésés. Le récepteur sigma-1 est principalement exprimé dans le cerveau et joue un rôle neuroprotecteur dans l'ischémie ou les maladies neurodégénératives.

Le récepteur sigma-1 est également exprimé dans des lignées cellulaires cancéreuses et des travaux récents suggèrent sa participation dans la prolifération et l'apoptose. Cependant, son rôle dans la carcinogénèse reste à découvrir.

Les canaux ioniques sont impliqués dans de nombreux processus physiologiques (rythme cardiaque, influx nerveux, …). Ces protéines membranaires émergent actuellement comme une nouvelle famille de cibles thérapeutiques dans les cancers. Au cours de ma thèse, j'ai montré que le récepteur sigma-1 régule l'activité du canal potassique voltage-dépendent hERG et du canal sodique voltage-dépendent Nav1.5 respectivement dans des cellules leucémiques et des cellules issues de cancer du sein. J'ai également montré que le récepteur sigma-1, à travers son action sur l'adressage du canal hERG, augmente l'invasivité des cellules leucémiques en favorisant leur interaction avec le microenvironnement tumoral.

Ces résultats mettent en évidence le rôle du récepteur sigma-1 sur la plasticité électrique des cellules cancéreuses et suggèrent l'intérêt de cette protéine chaperonne comme cible thérapeutique potentielle pour limiter la progression tumorale.

Summary :

The sigma-1 receptor is a chaperone protein active in damaged tissues. The sigma-1 receptor is mainly expressed into brain and have a neuroprotective role in ischemia and neurodegenerative diseases.

The sigma-1 receptor is also expressed into cancer cell lines and recent investigations suggest its involvement into proliferation and apoptosis. However, its role in carcinogenesis remains to delineating.

Ion channels are involved in numerous physiological processes (heart beating, nervous influx, …). These membrane proteins currently emerge as a new class of therapeutic targets in cancer. During my thesis, I observed that the sigma-1 receptor regulates voltage-dependent potassium channel hERG and voltage-dependent sodium channel $Na_v1.5$ activities respectively into leukemic and breast cancer cell lines. I also demonstrated that the sigma-1 receptor, through its action on hERG channel, increases leukemia invasiveness by promoting interaction with tumor microenvironment.

These results highlight the role of the sigma-1 receptor on cancer cell electrical plasticity and suggest this chaperone protein as a potential therapeutic target to limit tumor progression.

Abbréviations :

3-PPP : 3-(3-hydroxyphenyl)-N-propylpiperidine

ALS : sclérose latérale amyotrophique

AMPc : Adénosine-3', 5'-monophosphate cyclique

ARNm : Acide Ribonucléique messager

ASIC : canal ionique sensible au pH acide

ATP : Adénosine Tri-Phosphate

AVC : accident vasculaire cérébral

β1 : sous unité β1 des intégrines

BK_{Ca} : canal potassique à large conductance sensible au calcium

Ca_v : canal calcique voltage-dépendent

CFTR : Cystic Fibrosis Transmembrane conductance Regulator

DTG : 1,3-di-o-tolylguanidine

EAG : canal potassique Ether-A-Gogo

$E_{Na}C$: canal sodique épithélial

hERG : human Ether-a-gogo Related Gene

GIRK : G-protein Inwardly Rectifying K+ Channel

IL : interleukin

IP3 : Inositol 1,4,5 tri-phosphate

K_{2P} : canaux potassiques à deux boucles P

K_{Ca} : canaux potassiques sensibles au calcium

kDa : kiloDalton

K_{ir} : canaux potassiques à rectification entrante

KO : Knock-out

K_v : canal potassique voltage-dépendent

Na_v : canal sodique voltage-dépendent

NMDA : Acide N-Méthyl D-Aspartique

PCP : Phénylcyclidine

PLC : Phospholipase C

PKC : Protéine Kinase C

RE : réticulum endoplasmique

SCLC : Cancer du poumon à petites cellules

Sig1R : Récepteur Sigma-1

SK_{Ca} : canal potassique de faible conductance sensible au calcium

SOC : canaux calciques activés par la vidange du calcium du réticulum endoplasmique

TASK : TWIK-related Acidic-Sensitive K+ channel

TREK : TWIK-1-related K+ channel

TRP : Transient Receptor Potential channel

VEGF : Vascular Endothelial Growth Factor

VIH : virus de l'immunodéficience humaine

VRCC : courant chlorure régulé par le volume

Introduction : Le récepteur Sigma-1 – évolution d'un concept

A. La genèse :

L'origine du récepteur sigma remonte à la caractérisation des récepteurs opiacés, issue d'une étude comportementale réalisée chez le chien en 1976. Dans cette étude, les auteurs proposèrent d'attribuer les effets psychotomimétiques induits par trois agonistes morphiniques (la morphine, la ketocyclazocine et le SKF-10047) à l'existence de trois sous-types de récepteurs dénommés respectivement selon l'initiale du nom de la molécule : les récepteurs mu, kappa et sigma (Martin et al., 1976). Ainsi, le récepteur sigma fut assimilé pendant plusieurs années à un membre d'une famille de récepteurs couplés aux protéines G, les récepteurs opioïdes. A l'appui de cette hypothèse, les effets induits par le (+)-SKF-10047 sont bloqués par le naltrexone, un antagoniste à large spectre des récepteurs aux opiacés (Martin et al., 1976). Mais en 1982 et 1983, deux études visant à mieux caractériser le récepteur sigma, démontrèrent que le site de liaison du (+)-SKF-10047 n'était que très faiblement sensible aux agonistes et antagonistes des récepteurs aux opioïdes (morphine, naltrexone, naloxone, endorphines, enképhalines) (Su, 1982; Vaupel, 1983), excluant le site de liaison du (+)-SKF-10047 de la famille des récepteurs aux opiacés.

Dans la même étude, la phénylcyclidine (PCP) montre une certaine affinité envers le récepteur sigma de par sa capacité à entrer en compétition avec le (+)-SKF-10047 (Su, 1982). Cette molécule était alors connue pour interagir avec les récepteurs au glutamate de type NMDA (acide N-méthyl-D-aspartique) (Zukin et al., 1984). Ceci conduisit à proposer le récepteur opoïd/sigma comme un membre potentiel de la famille des récepteurs NMDA. Quelques années plus tard, on s'aperçut que la PCP et le (+)-SKF-10047 étaient capables, l'un comme l'autre, d'interagir avec le récepteur sigma et le récepteur de type NMDA avec des affinités différentes (Goldman et al., 1985; Zukin et al., 1986), expliquant ainsi la confusion entre les deux protéines.

En 1988, le terme de « récepteur sigma » fut finalement proposé pour différencier le site de liaison du (+)-SKF-10047 du récepteur opoïd/sigma postulé par Martin en 1976 (Quirion et al., 1992; Su et al., 1988). Vingt ans après la naissance du récepteur sigma, le clonage et le séquençage du gène correspondant l'ont définitivement exclu du groupe des récepteurs classiques (Hanner et al., 1996a; Mei and Pasternak, 2001; Seth et al., 1998). Observé dans différentes situations physiologiques et pathologiques, il fallut cependant attendre plus de 30 ans après sa conceptualisation pour obtenir les premières données sur

la fonction moléculaire du récepteur sigma-1 (Sig1R). En 2007, Hayashi et Su démontrent que lors de stress cellulaires (déplétion de glucose, vidange calcique du réticulum endoplasmique, choc thermique, …), le récepteur sigma-1 exerce une fonction de protéine chaperonne de canaux ioniques, protéines membranaires spécialisées dans le transport d'ions, contribuant à la survie cellulaire (Hayashi and Su, 2007). Cette fonction chaperonne du récepteur sigma-1 sera abordée plus en détail dans la troisième partie de cette introduction (III.C).

B. Les ligands sigma : un premier outil d'étude.

Considéré à tort comme un récepteur, le récepteur sigma fut longtemps étudié grâce à des approches pharmacologiques. Aussi, l'utilisation de ligands fut le premier moyen (et longtemps le seul) de cerner la fonction physiologique ou pathologique du récepteur sigma. Ceci engendrera le développement d'une large pharmacologie du récepteur sigma. Certains opioïdes (en particulier des benzomorphanes), alcaloïdes, stéroïdes, antidépresseurs et anticonvulsifs sont ainsi désignés comme des ligands sigma (Tableau 1). Ces molécules sont classées comme des « agonistes » ou des « antagonistes » selon qu'elles miment ou inhibent l'effet du (+)-SKF-10047 (ou d'autres molécules références telles que la (+)-pentazocine) dans des tests biologiques. Plus récemment, avec la découverte de la fonction chaperonne du récepteur sigma et de son interaction forte avec la protéine BiP (III.C), il a été proposé de revoir la classification des ligands sigma et ainsi de désigner comme « agonistes » tout composant capable de dissocier le récepteur sigma de la protéine BiP (Hayashi and Su, 2007; Katz et al., 2011).

Composé	Affinité	Sélectivité	Fonction
Benzomorphanes			
(+)-SKF-10047	+++	Sig1R	Agoniste
(+)-Pentazocine	+++	Sig1R	Agoniste
(+)-3-PPP	+++	Sig1R	Agoniste
Antipsychotiques			
Halopéridol	+++	Sig1R / Sig2R	Antagoniste
Nemonapride	+++	Sig1R / Sig2R	??

Antidépresseurs			
Imipramine	++	Sig1R	Agoniste
Fluoxetine	++	Sig1R	Agoniste
Fluvoxamine	+++	Sig1R	Agoniste
Igmésine (JO-1784)	+++	Sig1R	Agoniste
Neurostéroïdes			
Progestérone	+++	Sig1R	Antagoniste
Prégnénolone sulfate	+++	Sig1R	Agoniste
DHEA sulfate	++	Sig1R	Agoniste
Alcaloïdes			
Cocaïne	+	Sig1R	Agoniste
Met amphétamine	+	Sig1R	Agoniste
DMT	+	Sig1R	Agoniste
Autre composés			
DTG	+++	Sig1R / Sig2R	Agoniste
BD-1008	+++	Sig1R / Sig2R	Antagoniste
BD-1047	+++	Sig1R	Antagoniste
BD-1063	+++	Sig1R	Antagoniste
PRE-084	+++	Sig1R	Agoniste
NE-100	+++	Sig1R	Antagoniste
SA-4503	+++	Sig1R	Agoniste
Rimcazole	+	Sig1R	Antagoniste

Tableau 1 : Les ligands sigma (Adapté de Hayashi et al. 2004). Pour les valeurs de constante d'inhibition (Ki), +++ signifie < 50 nM, ++ signifie < 500 nM, + signifie < 10 µM. 3-PPP : 3-(3-hydroxyphényl-N-propylpiperidine, DTG : Ditolylguanidine ; DMT : N'N'-Dimethyltryptamine.

Certaines molécules sont également décrites comme des ligands endogènes de Sig1R. C'est le cas des neurostéroïdes et du N,N-diméthyltryptamine (DMT) (Fontanilla et al., 2009; Maurice et al., 1999). Malgré une affinité assez faible, ces ligands peuvent interagir avec Sig1R dans certaines conditions physiologiques (stress, apprentissage, mémoire, dépression, …).

C. Les autres récepteurs sigma :

Le développement des études pharmacologiques basées sur l'utilisation de ligands sigma exogènes a rapidement démontré l'existence d'une hétérogénéité des sites de liaison sigma. Le site de liaison sigma, initialement identifié, présente une forte affinité pour les (+)-benzomorphanes tels que le (+)-SKF-10047 ou la (+)-pentazocine, mais une faible affinité pour d'autres ligands sigma comme l'halopéridol ou le ditolylguanidine (DTG). Un autre site de liaison « sigma » de forte affinité pour le DTG et l'halopéridol, mais de plus faible affinité pour les (+)-benzomorphanes a été proposé comme un deuxième sous-type de récepteur sigma (Quirion et al., 1992). Ces deux types de récepteurs sigma furent respectivement dénommés récepteur sigma-1 (Sig1R) et récepteur sigma-2 (Sig2R).

Sig2R est notamment surexprimé dans de nombreux cancers et semble impliqué dans la prolifération cellulaire (Mach et al., 2013). Cette protéine est maintenant considérée comme un biomarqueur des cancers et plusieurs ligands de Sig2R réduisent la progression tumorale *in vivo* dans des modèles murins (Hornick et al., 2012; Niso et al., 2013; Zeng et al., 2012). D'un poids moléculaire de 19 kDa, la nature moléculaire de Sig2R est longtemps restée indéterminée. Mach et al. ont récemment proposé que Sig2R correspond en fait à la protéine Prgmc1, une sous unité d'un récepteur à la progestérone (Prgmc1) (Xu et al., 2011). Cependant, les conclusions de cette étude sont controversées puisque Prgmc1 présente un poids moléculaire de 25 kDa alors que le poids moléculaire de la protéine qui porte le site de liaison des ligands sigma 2 est de 19 kDa (Ruoho A.E. et al, 2013 – Neurosciences Meeting).

Un troisième sous-type de récepteur Sigma, le récepteur sigma-3 (Sig3R), a été proposé sur la base d'une faible affinité pour le DTG et d'une activation induite par l'halopéridol, le spipérone et certains phtalènes. Sig3R serait impliqué dans la synthèse de la dopamine dans le striatum (Booth et al., 1999; Booth et al., 1993; Myers et al., 1994). Cependant, la nature et les fonctions de ce troisième sous-type sont très peu documentées

et les recherches sur ce nouveau type de récepteur sigma semblent ne pas avoir été poursuivies.

Mon projet de thèse se focalise sur l'étude du récepteur sigma-1 (Sig1R) dont je détaillerai les fonctions physiopathologiques et moléculaires dans les parties suivantes.

D. La distribution tissulaire du récepteur Sigma-1 :

Sig1R présente une distribution relativement ubiquitaire. Néanmoins, la densité d'expression est plus élevée dans certains tissus tels que le cerveau, le foie, le cœur, le système gastro-intestinal, la rate, les reins et certains composants du système immunitaire (Bouchard et al., 1997; Ela et al., 1994; Harada et al., 1994; Hellewell et al., 1994; Su, 1982; Su et al., 1988; Wolfe et al., 1997) (Figure 1).

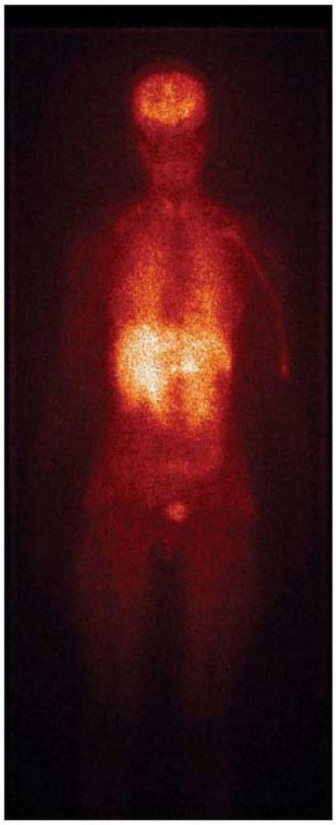

Figure 1 : Distribution humaine de la fixation d'un ligand sigma-1 de haute affinité marqué à l'iode radioactif, [123I]TPCNE, sur un sujet sain (d'après Stone et al. 2006). Cette image est représentative de quatre expériences identiques. On note principalement un marquage au niveau du cerveau et du foie ainsi qu'un marquage plus modéré au niveau des poumons.

Dans le cerveau, on détecte Sig1R de façon discrète dans le cortex cérébral (frontal et cingulaire), l'hippocampe (cellules granulaires du gyrus denté et neurones pyramidaux de la corne d'Ammon), l'hypothalamus, le thalamus, le cervelet (corps cellulaires de Purkinje et noyaux profonds), le mésencéphale (noyau rouge) et le noyau accumbens (Bouchard et al., 1997; Seth et al., 1998; Walker et al., 1990) (Figure 2).

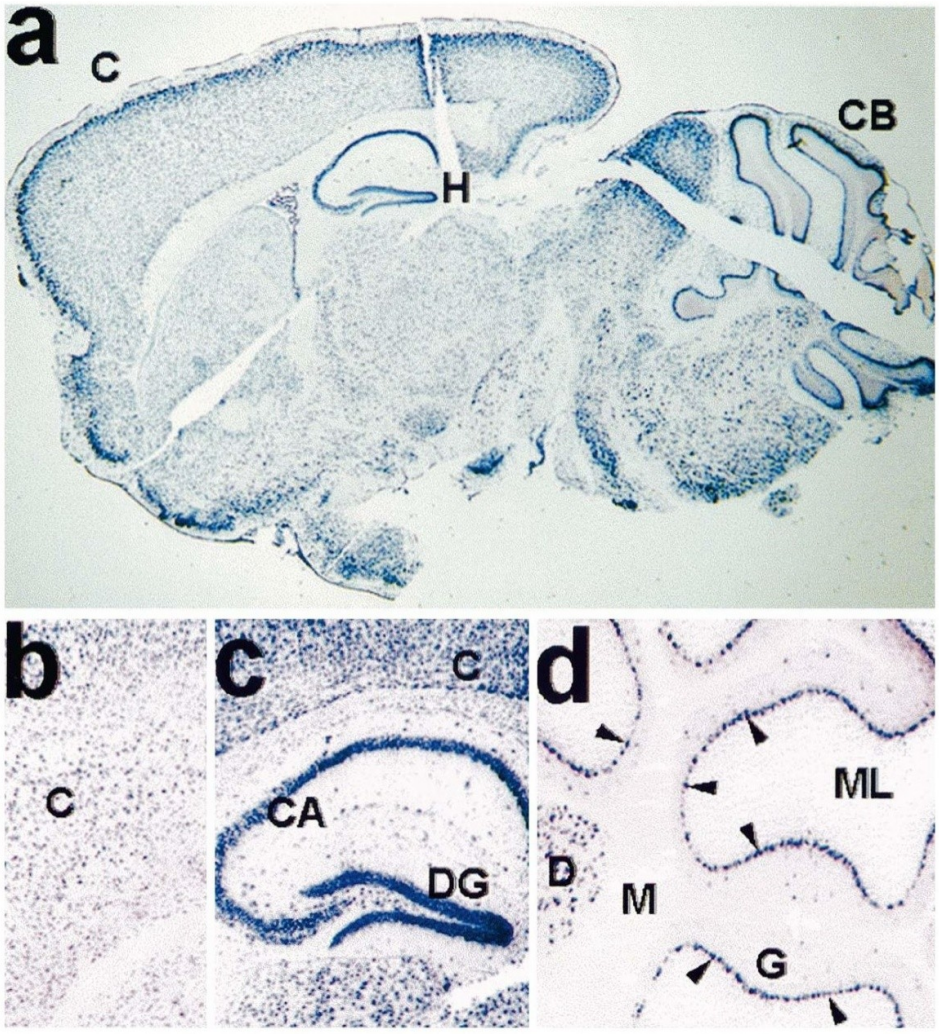

Figure 2 : Hybridation in situ pour l'analyse de la distribution de l'ARNm codant pour Sig1R dans le cerveau de souris (d'après Seth et al. 2001). a) coupe sagittale du cerveau de souris adulte hybridée avec une sonde antisens dirigée contre l'ARNm de Sig1R (C : cortex, H : hippocampe, CB : cervelet). b) Grossissement du cortex cérébral. c) Grossissement de l'hippocampe (C : cortex cérébral, CA : corne d'Ammon, DG : Gyrus denté). d) Grossissement du cervelet (G : couche granulaire, ML : couche moléculaire, M : medulla de la substance blanche, D : Noyau cérébelleux profond, flèches : corps cellulaires de Purkinje).

Ces observations, réalisées dans plusieurs modèles d'étude (souris, rat, porc, homme) à l'aide de différentes techniques (tels que l'hybridation *in situ* d'ARN messagers

spécifiques de la séquence de Sig1R, de ligands radiomarqués ou d'anticorps) ont permis de suggérer le rôle de Sig1R dans certaines fonctions associées à ces structures.

II. Les fonctions de Sig1R

Historiquement, Sig1R a été relié à certains troubles psychotiques, notamment ceux induits par des molécules de la famille des benzomorphanes. De plus, la distribution de Sig1R dans certaines régions du cerveau a suggéré son implication dans des fonctions physiologiques telles que la mémoire, l'apprentissage ou la douleur et dans des pathologies telles que l'amnésie, la maladie d'Alzheimer, la dépression et l'addiction (pour revues : (Hayashi and Su, 2005; Katz et al., 2011; Maurice and Su, 2009)).

A. La mémoire

Sur des modèles de souris rendues amnésiques à l'aide d'inhibiteurs des récepteurs à l'acétylcholine, des ligands sigma tels que la pentazocine ou le (+)-SKF-10047 soutiennent les processus mnésiques en favorisant chacune des étapes de la mémoire (acquisition, consolidation, rétention) (Earley et al., 1991; Matsuno et al., 1996; Matsuno et al., 1993; Maurice et al., 1994a; Maurice et al., 1994b; Maurice et al., 1991; Urani et al., 1998). D'autres agonistes de Sig1R réduisent l'amnésie induite par l'inhibition des neurones glutaminergiques (induite par un antagoniste des récepteurs aux NMDA) de l'hippocampe (Maurice et al., 1994b; Monnet et al., 1990).

Ainsi, Sig1R – ou plutôt l'action des agonistes de Sig1R – régule l'activité des neurones cholinergiques et glutaminergiques et favorise les processus d'apprentissage et de mémorisation. Néanmoins, les effets des ligands sigma ne sont pas observés en absence de stimuli pro-amnésiques. Cela suggère que Sig1R n'agit pas directement sur les processus de mémorisation et d'apprentissage mais exerce plutôt un rôle protecteur sur ces processus lorsque leur intégrité est affectée.

Dans le contexte du vieillissement, les ligands sigma réduisent également la perte de mémoire et augmentent la capacité d'apprentissage (Maurice et al., 1996). Il est suggéré que la perte de mémoire observée au cours du vieillissement est liée à l'expression de certains neurostéroïdes. Les neurostéroïdes ont un rôle important dans les processus mnésiques et anti-amnésiques (Monnet and Maurice, 2006). La prégnénolone, précurseur des hormones

stéroïdiennes, est un ligand sigma (Monnet et al., 1995). Cette molécule fortement produite chez les individus jeunes, l'est de moins en moins au cours du vieillissement de l'organisme (Baulieu et al., 2001). Ainsi, la diminution de production de prégnénolone au cours du vieillissement peut entrainer entre autre une réduction de son effet activateur sur Sig1R. Par conséquent, Sig1R étant de moins en moins actif, les conditions sont de plus en plus favorables à l'apparition d'amnésies. Ainsi, la perte de l'expression de la prégnénolone pourrait ainsi être compensée par l'utilisation d'agoniste sigma exogène pour renforcer les processus mnésiques chez le sujet âgé et mimer l'action des neurostéroïdes sur Sig1R (Hayashi and Su, 2004a; Monnet and Maurice, 2006). Apportant du crédit à cette hypothèse, plusieurs études ont montré que certains neurostéroïdes (prégnénolone et DHEA) ont un effet pro-mnésique bloqué par des antagonistes de Sig1R (NE-100, progestérone) (Urani et al., 1998; Yang et al., 2012b).

Les maladies neurodégénératives telles que la maladie d'Alzheimer sont une cause importante de déficits mnésiques. En effet, le premier symptôme de cette maladie neurodégénérative est la perte de mémoire. De la même manière que pour le vieillissement, le développement de la maladie s'accompagne d'une diminution de la production de prégnénolone et d'autres neurostéroïdes par le cerveau (Mayo et al., 2001; Weill-Engerer et al., 2002). On peut donc supposer, comme dans le cadre du vieillissement, que la prégnénolone à travers son action sur Sig1R peut être importante pour retarder le développement de la maladie. Dans des modèles murins, on constate par ailleurs que les ligands sigma (dont la prégnénolone et le DHEA) ralentissent le développement de la maladie d'Alzheimer en empêchant notamment la mort neuronale induite par la protéine amyloïde β (Marrazzo et al., 2005; Maurice et al., 1998; Meunier et al., 2006; Villard et al., 2009; Yang et al., 2012b). Alors que l'expression de Sig1R est stable tout au long de la vie de la souris (Phan et al., 2003), l'expression de Sig1R est réduite dans les cerveaux de patients souffrant de la maladie d'Alzheimer, suggérant un rôle de cette protéine dans les mécanismes de protection de la mémoire (Hedskog et al., 2013; Jansen et al., 1993; Mishina et al., 2008).

Parallèlement aux études pharmacologiques basées sur l'utilisation de ligands sigma, des études génétiques ont mis en évidence chez l'Homme l'existence de certaines variations dans la séquence du gène de Sig1R – ce que l'on appelle des polymorphismes sont significativement associés à l'apparition de la maladie d'Alzheimer (Feher et al., 2012; Huang et al., 2011). L'une de ces études démontre une corrélation entre l'apparition du polymorphisme Q2P (A61C) de Sig1R avec le variant du gène codant pour l'apolipoprotéine

E, connu pour conférer une grande prédisposition des porteurs à la maladie d'Alzheimer (Huang et al., 2011). La seconde étude indique que ce même polymorphisme ainsi qu'un autre polymorphisme de Sig1R (G240T/C241T) sous la forme de l'haplotype TT-P (forme du gène de Sig1R portant les deux polymorphismes) est significativement associé à l'apparition de la maladie d'Alzheimer. Cette étude montre également une interaction significative avec le variant du gène de l'apolipoprotéine E (Feher et al., 2012). Néanmoins, deux autres études font état soit d'une absence de corrélation significative de ces polymorphismes avec l'apparition de la maladie d'Alzheimer (Maruszak et al., 2007), soit d'un effet protecteur de l'haplotype TT-P contre l'apparition de la maladie d'Alzheimer (Uchida et al., 2005). Il est intéressant de remarquer que l'haplotype TT-P conduit à une réduction de l'expression de Sig1R (Miyatake et al., 2004). Aussi, on peut s'interroger sur la pertinence de la conclusion d'Uchida et al. – une plus faible expression de Sig1R conduit à une probabilité plus faible de développer la maladie d'Alzheimer – qui est en complète opposition avec les travaux publiés précédemment démontrant l'effet protecteur des agonistes de Sig1R et la corrélation négative de l'expression de la protéine Sig1R avec l'apparition de la maladie d'Alzheimer (Jansen et al., 1993; Maurice et al., 1998).

A l'heure actuelle, bien que la piste soit passionnante, il est difficile de conclure quant à la réelle implication des polymorphismes de Sig1R dans le développement de la maladie d'Alzheimer. Néanmoins, il semble clair que Sig1R a une action protectrice – à travers son expression et/ou l'action de ces ligands – contre le développement de cette maladie.

B. La dépression

La dépression est une maladie psychiatrique importante qui touche près de 8% de la population française (Chan Chee et al., 2009 - INPES). Cette maladie peut être biologiquement associée à différentes perturbations du système nerveux central. Elle peut être liée à un déficit dans la libération de certains neurotransmetteurs (sérotonine, dopamine, norépinephrine), à des anomalies structurales du cerveau (notamment du cervelet, de l'hippocampe, du thalamus ou des lobes frontaux) ou encore à une perte de neurones de l'hippocampe menant à des dysfonctionnements de la mémoire et des troubles de l'humeur (Kempton et al., 2011; McIntosh et al., 2011; Nutt, 2008).

Le rôle de Sig1R dans la dépression a été rapidement révélé. En effet, certains antidépresseurs tels que la Sertraline, la Clorgyline et la Fluvoxamine) interagissent avec Sig1R (Bergeron et al., 1993; Narita et al., 1996). De façon corollaire, des ligands sigma 1 de

référence comme la (+)-Pentazocine, le (+)-SKF-10047, l'Igmésine, le SA-4503, le DHEA ou le PRE-084 présentent des effets antidépresseurs *in vivo* chez la souris dans des tests de nage forcée (Maurice et al., 2001; Reddy et al., 1998; Urani et al., 2001). Plusieurs articles ont par la suite montré que les ligands Sig1R agissent respectivement sur les neurones sérotoninergiques et glutaminergiques des noyaux raphés et de l'hippocampe (Bermack and Debonnel, 2001, 2005) et peuvent expliquer le rôle de Sig1R dans l'apparition de la dépression.

Ces différentes études ont conforté l'idée que Sig1R est une cible potentiellement prometteuse pour établir de nouveaux antidépresseurs. Dans ce sens, l'Igmésine – extensivement décrite comme un antidépresseur efficace *in vivo* chez l'animal (Bermack et al., 2002; Ukai et al., 1998; Urani et al., 2001; Urani et al., 2004) – est le seul ligand sigma à avoir été testé en phase clinique dans le cadre de la dépression (Volz and Stoll, 2004). Dans une condition particulière (25 mg / kg), l'Igmésine a montré un effet anti-dépresseur significatif sur une cohorte de patients. Cependant, la globalité de l'étude conclut sur un effet non-significatif de l'Igmésine sur la dépression. Son développement commercial fut arrêté.

Plus récemment, une souris dont le gène codant pour Sig1R est invalidé, a été développée (Langa et al., 2003). Cette souris Sig1R KO constitue un nouvel outil intéressant pour explorer la fonction de Sig1R tout en s'affranchissant de l'usage de ligands « activateur » ou « inhibiteur » de Sig1R. Au vu des résultats obtenus avec les ligands, on s'attendait à ce que cette souris Sig1R KO soit beaucoup plus déprimée que la souris sauvage exprimant Sig1R. Etonnamment, la souris Sig1R KO ne présente ni d'altération du comportement moteur ni d'hyper-anxiété au regard d'une souris « classique ». Mais lors du test de nage forcée, la souris Sig1R KO présente une immobilité plus importante que la souris « sauvage » exprimant Sig1R, traduisant un état dépressif plus important (Sabino et al., 2009).

Ainsi, l'activation de Sig1R a un rôle important pour inhiber le développement des symptômes dépressifs en régulant notamment les voies glutaminergiques et sérotoninergiques. L'action positive des agonistes de Sig1R et les essais précliniques de l'Igmésine sont en accord avec cette proposition. Chez la souris Sig1R KO, l'absence de symptômes dépressifs en conditions basales montre que l'inhibition de Sig1R ne participe pas directement à l'apparition de la dépression. Par contre, l'observation d'un comportement dépressif dans les tests de « nage forcée » démontre que l'inhibition de Sig1R favorise l'état dépressif lorsque le sujet se retrouve confronté à une situation stressante.

C. La dépendance et l'addiction

A travers l'action du (+)-SKF-10047, l'un des premiers rôles attribué au récepteur sigma-1 a été son implication dans l'apparition de troubles psychotiques. Ajouté à cela, une grande partie des ligands sigma identifiés sont considérés comme des agents psychotropes et sont pour certains classés comme substances addictives tels que la cocaïne, la méthamphétamine, l'alcool ou le dextrométorphane. Ainsi, c'est tout naturellement que la fonction de Sig1R a été étudiée dans le contexte de l'addiction. Je présenterai ici l'addiction à deux substances addictives pour lesquelles le rôle de Sig1R a été particulièrement bien décrit, la méthamphétamine et la cocaïne.

1. La méthamphétamine

Chez l'homme, la méthamphétamine induit une anxiété, une agitation, parfois une paranoïa, des hallucinations, une dépression et des troubles du sommeil. Chez la souris, la méthamphétamine induit un comportement particulier caractérisé par une augmentation de l'activité locomotrice puis par l'apparition de mouvements répétitifs et convulsifs (reniflement, mordillements, balançage de la tête, déplacements en rond) que l'on appelle stéréotypie.

La méthamphétamine a été décrite comme étant un agoniste de Sig1R (Nguyen et al., 2005). Certaines caractéristiques du comportement induit par la méthamphétamine sont modulées lorsque l'animal est traité par des antagonistes de Sig1R, BMY-14802 et BD-1047, sans pour autant affecter la quantité totale de mouvements stéréotypiques observés (Kitanaka et al., 2009). Les antagonistes de Sig1R, MS-377 et BD-1047 sont capables de bloquer la sensibilisation à la méthamphétamine (Nguyen et al., 2005; Takahashi et al., 2001). Mais l'activité de Sig1R n'est pas le seul paramètre modulé par la méthamphétamine. En effet, une étude a montré que le mésencéphale de souris conditionnée à « s'auto-administrer » de la méthamphétamine présente une plus forte expression de Sig1R comparé à celui de souris auxquels la méthamphétamine est injectée de façon passive (Stefanski et al., 2004). Ceci suggère que la souris « dépendante » de la méthamphétamine façonne son mésencéphale de façon à exprimer une plus forte densité de Sig1R. On peut imaginer que cette surexpression de Sig1R permet à l'animal de mieux satisfaire (et entretenir) sa dépendance en augmentant le nombre de sites disponibles pour la méthamphétamine.

Ainsi, Sig1R est un acteur important dans la plasticité et le remodelage cérébral induit par la dépendance à la méthamphétamine. Les antagonistes de Sig1R pourraient ainsi être

proposés comme substances thérapeutiques pour soigner les addictions à la méthamphétamine. Néanmoins, il est important de préciser que tous les antagonistes n'ont pas exactement les mêmes impacts sur les effets comportementaux induits par la méthamphétamine, ce qui laisse supposer que la relation qui lie Sig1R et la méthamphétamine est complexe et nécessite une certaine clarification avant d'envisager des tests cliniques.

2. La cocaïne

Comme pour la méthamphétamine, l'effet de la cocaïne sur Sig1R a été intensivement étudié depuis plusieurs décennies et a permis de mieux comprendre la fonction primaire de Sig1R. Cette drogue très populaire est un alcaloïde connu pour ses effets anesthésiants (ce fut sa première utilisation), euphorisants mais également psychotiques, dépressifs et surtout addictifs (Bernfeld, 1953; Matas, 1952). Ses effets résultent en partie de son action inhibitrice sur la recapture des monoamines (tels que la dopamine) par leurs récepteurs (Izenwasser, 2004; Riddle et al., 2005).

En 1982, Su suggère que la cocaïne est un faible agoniste du récepteur sigma (Su, 1982). En 1983, après que différents ligands sigma aient montré une certaine efficacité dans l'apparition de symptômes addictifs (en particulier sur le caractère de renforcement de la prise volontaire de ces molécules), une étude a comparé les effets des ligands sigma avec ceux induits par la PCP dans un modèle de substitution de l'addiction à la cocaïne (Slifer and Balster, 1983). Les auteurs observent notamment que le (+)-SKF-10047 induit des effets similaires à ceux de la cocaïne sur le renforcement de l'addiction des animaux et qu'en absence de cocaïne, le (+)-SKF-10047 préserve certaines caractéristiques addictives induites par la cocaïne. Cette toute première étude montre que des ligands sigma peuvent être des substituts de la cocaïne et que par ailleurs, certains symptômes dus à la cocaïne sont la résultante de son interaction avec Sig1R.

Pourtant, l'affinité de la cocaïne pour Sig1R n'est que de 2 mM (pour référence, le (+)-SKF-10047 a une affinité de 10 nM pour Sig1R, soit 400 000 fois plus affin) (Sharkey et al., 1988). En dépit de cette faible affinité, plusieurs études ont montré que, l'injection d'antagonistes de Sig1R ou de séquences anti-sens, ciblant le gène Sig1R (et donc empêchant son expression) avant celle de la cocaïne, peuvent bloquer certains de ses effets tels que l'hyperlocomotion, la désensibilisation, le renforcement positif, le conditionnement de préférence de place ou encore la dépendance (Martin-Fardon et al., 2007; Matsumoto et

al., 2002; Romieu et al., 2000; Romieu et al., 2002). Ces études ont montré que l'activité et l'expression de Sig1R sont nécessaires à l'établissement d'un profil addictif à la cocaïne.

De façon corollaire, la cocaïne influe également sur le niveau d'expression de Sig1R, l'augmentant dans les régions du cerveau liées à l'addiction et à la récompense (cortex, striatum, hippocampe) (Liu et al., 2005; Liu and Matsumoto, 2008). Cette augmentation d'expression de Sig1R est directement corrélée à l'apparition des symptômes comportementaux induits par la cocaïne (Liu and Matsumoto, 2008).

A l'instar de ce qui a été observé pour la méthamphétamine, ceci suggère que la cocaïne, en partie à travers son action sur Sig1R, induit un remodelage de certaines zones du cerveau (liées à l'addiction) entrainant une surexpression de Sig1R, augmentant en retour les effets de la cocaïne. Soutenant cette hypothèse, une étude récente montre que la cocaïne induit, via Sig1R, un remodelage de l'activité électrique des neurones du noyau accumbens et favorise par ce mécanisme la dépendance vis-à-vis de la cocaïne (Kourrich et al., 2013).

Au final, Sig1R apparait comme un élément important dans l'établissement de l'addiction à la cocaïne ou la méthamphétamine. Ceci suggère que des antagonistes de Sig1R pourraient être de bonnes solutions thérapeutiques pour traiter les personnes souffrant d'addiction à ces drogues. De façon plus fondamentale, ce rôle pathologique de Sig1R a surtout permis de mettre en évidence son implication dans la plasticité cérébrale, ouvrant de nouvelles perspectives de recherches pour comprendre les fonctions de Sig1R dans le cerveau.

D. L'accident vasculaire cérébral (AVC)

L'accident vasculaire cérébral (AVC) est une attaque cérébrale qui peut être d'origine ischémique ou hémorragique. L'AVC ischémique prive subitement les neurones d'oxygène et de glucose entrainant rapidement leur mort (il est estimé que 2 millions de neurones meurent chaque minutes que dure un AVC). Dans le cas d'un AVC hémorragique, la formation d'un hématome et d'un œdème augmente la pression intracrânienne conduisant ainsi à un écrasement des neurones et des déchirures de leurs axones.

Dans ce cadre pathologique, plusieurs études ont révélé un rôle neuroprotecteur de Sig1R. En effet, l'utilisation de ligands sigma chez des souris ayant subi une occlusion artérielle (mimant ainsi l'AVC ischémique) réduit le volume de l'infarctus dans le cortex, diminue l'inflammation et améliore la survie des neurones (Ajmo et al., 2006; Cuevas et al., 2011a; Goyagi et al., 2001; Hall et al., 2009b; Harukuni et al., 2000; Leonardo et al., 2010; Luedtke et al., 2012; Ruscher et al., 2012; Ruscher et al., 2011; Schetz et al., 2007; Shen et al., 2008).

L'effet protecteur de Sig1R trouve son origine à travers plusieurs mécanismes. Premièrement, les ligands sigma réduisent la toxicité induite par l'oxyde nitrique produit par les zones ischémiées au cours d'un AVC (Ajmo et al., 2006; Vagnerova et al., 2006). Dans les neurones, les ligands sigma réduisent la signalisation calcique induite par l'ischémie ou l'acidose (Cuevas et al., 2011a; Herrera et al., 2008; Shen et al., 2008). Par ailleurs, Sig1R semble également nécessaire à l'initiation de la repousse des axones lésés en permettant le remodelage de la membrane plasmique de ces cellules (Ruscher et al., 2011). Enfin, dans les cellules microgliales, les agonistes de Sig1R permettent également d'inhiber l'initiation de la réponse inflammatoire induite par les débris cellulaires résultant de l'AVC, en particulier en inhibant la signalisation calcique induite par les fortes concentrations d'ATP extracellulaires provenant de la destruction des neurones proches (Cuevas et al., 2011b).

Cet effet de Sig1R sur l'AVC est cependant à nuancer puisque plusieurs études observent que l'effet protecteur de Sig1R n'est que temporaire et permet seulement de retarder les lésions induites par l'AVC (Hall et al., 2009c; Leonardo et al., 2010).

E. La douleur

La douleur est un message d'alerte généré lorsque l'intégrité tissulaire est rompue. Ce message, produit par les récepteurs nociceptifs, est reçu et interprété par le cortex cérébral et permet de préserver l'individu en l'alertant sur l'intégrité de son organisme.

Sig1R est un acteur moléculaire particulièrement intéressant pour moduler la douleur (Zamanillo et al., 2013). En effet, l'inhibition de l'expression de Sig1R (à l'aide de sondes anti-sens) ou l'utilisation d'antagonistes permettent de favoriser les effets analgésiques de la morphine et d'autres opioïdes (Sanchez-Fernandez et al., 2014). De façon corollaire, l'activation de Sig1R par la (+)-pentazocine diminue le pouvoir analgésique de ces mêmes

molécules (Mei and Pasternak, 2002, 2007). Plus récemment, des résultats similaires ont été observés pour des douleurs neuropathiques ou inflammatoires (Nieto et al., 2014; Parenti et al., 2014; Tejada et al., 2014; Vidal-Torres et al., 2014).

Il est intéressant de noter que comme pour l'amnésie et la dépression, l'utilisation de ligands sigma seuls, ou l'inhibition de l'expression de Sig1R, n'induit pas de douleurs ce qui renforce l'idée que Sig1R est un modulateur neuronal actif dans des situations particulières mais silencieux en temps normal (Diaz et al., 2009; Nieto et al., 2014; Romero et al., 2012).

Ainsi, ces études, qui au départ étaient destinées à utiliser Sig1R dans le traitement de la dépendance aux opioïdes plutôt que dans le cadre de la douleur, ont permis de susciter un intérêt de la part de la communauté scientifique pour cette fonction physiologique de Sig1R. Cela a conduit au développement de nouveaux antagonistes de Sig1R, proposés comme antidouleurs. Actuellement, l'antagoniste S1RA est en cours de tests cliniques et a montré de bons résultats dans la phase I (Abadias et al., 2013).

F. La schizophrénie

De par son lien avec les molécules psychotropes, Sig1R a été étudié dans le contexte de la schizophrénie. Plusieurs ligands sigma ont été développés et testés sur des patients souffrants de schizophrénie. Malgré quelques résultats encourageants sur les symptômes négatifs de la schizophrénie (anxiété, dépression, …), ces molécules n'ont montré que peu ou pas d'efficacité sur les symptômes positifs (hallucinations, troubles cognitifs, etc …), certains même avaient l'effet inverse et augmentaient les symptômes positifs de la maladie (pour revue : (Hayashi et al., 2011; Niitsu et al., 2012).

L'expression de Sig1R est cependant altérée chez les patients atteints de schizophrénie. En effet, deux études ont rapporté une réduction de l'expression de Sig1R (à l'aide de ligands radiomarqués) dans le cervelet et le cortex temporal, frontal et occipital ainsi qu'une augmentation dans le cortex cingulaire (Shibuya et al., 1992; Weissman et al., 1991). Ceci suggère donc un rôle potentiel de l'expression de Sig1R dans la schizophrénie.

En parallèle à ces différences du profil d'expression de Sig1R, deux études affirment que l'haplotype TT-P et le polymorphisme Q2P de Sig1R sont des indicateurs de la prédisposition à la schizophrénie (Ishiguro et al., 1998; Ohi et al., 2011). Mais trois autres

études ne sont pas parvenues aux même conclusions et n'observent pas de corrélation entre les polymorphismes de Sig1R et la schizophrénie, jetant ainsi un doute raisonnable quant à la significativité de cette hypothétique association (Satoh et al., 2004; Takizawa et al., 2009; Uchida et al., 2003)).

Aussi, le rôle de Sig1R dans la schizophrénie n'est pas encore compris. Dans le même temps, cette maladie mentale est elle-même encore mal comprise. De nouvelles études à la fois sur Sig1R et sur les mécanismes à l'origine de la schizophrénie devraient permettre de mieux comprendre la place de Sig1R dans cette maladie.

G. La sclérose latérale amyotrophique

La sclérose latérale amyotrophique (ALS) ou maladie de Charcot est une maladie neurodégénérative grave qui touche principalement les motoneurones. Elle se caractérise par une paralysie progressive qui altère l'ensemble du système musculaire des membres et le tronc cérébral. Environ 5% des patients souffrant d'ALS développent également des démences fronto-temporal (DFT) (maladie neurologique qui induit une atrophie des lobes cérébraux). Les causes de l'ALS sont encore mal connues. Actuellement, plusieurs hypothèses ont été émises pour expliquer cette maladie. La principale hypothèse repose sur une dérégulation de la super-oxyde dismutase (SOD1) qui crée un stress oxydatif menant à la destruction des neurones. Dans ce sens, une mutation de la SOD1 a été retrouvée chez 20% des patients atteints d'ALS héréditaires et 5% de ceux atteints d'ALS sporadiques. Un défaut des voies cholinergiques ou glutaminergiques est également suspecté d'être responsable du développement de l'ALS (Spalloni et al., 2013).

Contrairement aux autres situations physiologiques et pathologiques dans lesquelles Sig1R a été étudié, dans le cas de l'ALS, les premières études n'étaient pas des études pharmacologiques mais génétiques. En effet, plusieurs mutations du gène codant Sig1R ont été observées chez des patients souffrant d'ALS. La première d'entre elle est une mutation présente sur la partie 3'-UTR du gène SIGMAR1 (c.672*51G>T) qui entraine une surexpression de la protéine Sig1R. Les auteurs concluent que cette surexpression de Sig1R désorganise la régulation génique du neurone en excluant certaines protéines nucléaires (TDP53 et FUS) du noyau (Luty et al., 2010). Une autre mutation sur le premier segment transmembranaire (E102Q) de Sig1R est également observée chez une famille souffrant d'ALS juvénile héréditaire. Cette mutation rend les cellules plus sensibles à la mort cellulaire (Al-Saif et al., 2011). Mais récemment une nouvelle étude a remis ces résultats en question.

En effet, sur une cohorte de 25 cas d'ALS héréditaires et une cohorte témoins de 380 personnes, les auteurs n'ont pas pu relier la mutation 3'-UTR de SIGMAR1 avec l'apparition de l'ALS ou de la DFT (Belzil et al., 2013).

Plusieurs études ont montré une diminution de l'expression de Sig1R lors du développement de l'ALS (Mavlyutov et al., 2013; Prause et al., 2013). Dans ces études, les auteurs observent que cette réduction de l'expression de Sig1R est liée à la mutation SOD1. Cette perte de Sig1R entraine une hyperexcitabilité des motoneurones cholinergiques post-synaptique de la corde spinale (Mavlyutov et al., 2013). Par ailleurs, dans des cellules de patients souffrant d'ALS, Sig1R semble anormalement localisé et forme des agrégats dans le RE à l'interface avec le protéasome associé au RE. Cette accumulation provoque des dysfonctionnements de la machine de synthèse et de dégradation des protéines, de gestion des réponses au stress (UPR, système permettant à la cellule d'éviter la mort cellulaire en régulant un défaut de synthèse des protéines) et de la signalisation calcique du neurone. L'inhibition de l'expression de Sig1R dans des cellules murines (NSC-34) mime les effets observés dans les cellules de patients souffrant d'ALS. Ainsi, à travers ces mécanismes, l'inhibition de l'expression de Sig1R et sa localisation anormale favorise l'apoptose des neurones et donc la progression de la maladie (Prause et al., 2013).

Pour finir, certains agonistes de Sig1R (PRE-084 et SA4503) ont montré des effets neuroprotecteurs dans des modèles de souris développant spontanément l'ALS (Mancuso et al., 2012; Ono et al., 2014; Peviani et al., 2014).

Ainsi, l'expression de Sig1R jouerait un rôle neuroprotecteur contre le développement de l'ALS et l'utilisation de ligands sigma pourrait permettre d'améliorer la survie des patients.

H. L'immunité et le VIH

Identifié dans le système immunitaire (Wolfe et al., 1997), Sig1R a été essentiellement étudié à travers l'action de la cocaïne sur la progression du virus de l'immunodéficience humaine (VIH). En effet, la cocaïne favorise la réplication du VIH dans les leucocytes. Ainsi, il a été observé que la cocaïne, à travers son action sur Sig1R, entraine un remodelage du profil d'expression des récepteurs au chimiokines (principalement dédiés à la migration des cellules de l'immunité dans l'organisme). Ce remodelage (surexpression des CCR5 et CXCR4) augmente le pouvoir infectieux du VIH puisqu'il utilise

ces récepteurs comme porte d'entrée pour infecter les cellules de l'immunité (Roth et al., 2005). Des résultats similaires ont été obtenus sur la microglie (cellules immunitaires présentes dans le tissu cérébral) (Gekker et al., 2006). Enfin, deux études ont montré récemment que la cocaïne, en activant Sig1R, induit l'expression de différentes molécules d'adhésion qui facilitent la migration des monocytes à travers la barrière hémato-encéphalique, pouvant ainsi expliquer certains désordres neurologiques induits par le VIH (Yao et al., 2011; Yao et al., 2010).

Au-delà du contexte particulier de l'immunité anti-VIH associée à la consommation de cocaïne, Sig1R a été impliqué dans l'activation microgliale. Ainsi, certains agonistes de Sig1R inhibent la réponse inflammatoire de la microglie à la présence d'éléments pathogènes (lipopolysaccharide) ou de molécules pro-inflammatoire (ATP) en agissant principalement sur l'inhibition de la signalisation calcique de ces cellules (Cuevas et al., 2011b; Hall et al., 2009a).

Bien que Sig1R soit observé dans les zones riches en lymphocytes T et B de la rate, la majorité des études ont montré un effet fonctionnel de Sig1R dans la microglie. Des études complémentaires seront nécessaires afin de déterminer si Sig1R est présent et participe à l'action des autres acteurs du système immunitaire (lymphocytes, cellules dendritiques, cellules NK, …).

I. Les cancers

Brièvement, les cancers se définissent par une transformation aberrante (par mutations ou altérations génétiques) de certaines cellules au sein d'un tissu menant à une prolifération anarchique de ces cellules et la formation d'une tumeur. Cette prolifération anarchique crée les conditions favorables à d'autres altérations génétiques qui permettent à la tumeur d'évoluer et d'acquérir de nouvelles capacités telles que la résistance accrue à la chimiothérapie ou la formation de tumeurs secondaires ou métastases. Un certain nombre de ces capacités « pro-tumorales » ont été isolées et présentées comme les caractéristiques des cellules cancéreuses (« hallmarks » du cancer (Hanahan and Weinberg, 2000, 2011)) (Figure 3).

Tout d'abord limité à six fonctions majeures de la progression tumorale, ces dernières années ont vu l'émergence de quatre nouveaux « hallmarks » que sont la dérégulation du

métabolisme des cellules cancéreuses, leur instabilité génomique, l'inhibition de leur destruction par le système immunitaire et leur capacité à promouvoir un environnement pro-inflammatoire (Figure 3). Ces deux derniers « hallmarks » traduisent une évolution de la recherche scientifique sur le cancer au cours des dernières années. En effet, la tumeur n'est plus simplement vue comme un simple agglomérat de cellules cancéreuses proliférant de façon anarchique mais plutôt comme un tissu comprenant des cellules cancéreuses et non-cancéreuses (fibroblastes, cellules immunes, adipocytes) et d'autres éléments extracellulaires (matrice extracellulaire, facteurs de croissance, cytokines, etc …). L'ensemble de ces éléments constitue le microenvironnement tumoral. La cellule tumorale interagit constamment avec chacun de ces éléments du microenvironnement et les « utilise » afin de promouvoir sa survie, sa croissance, sa propagation ou encore sa résistance à la chimiothérapie.

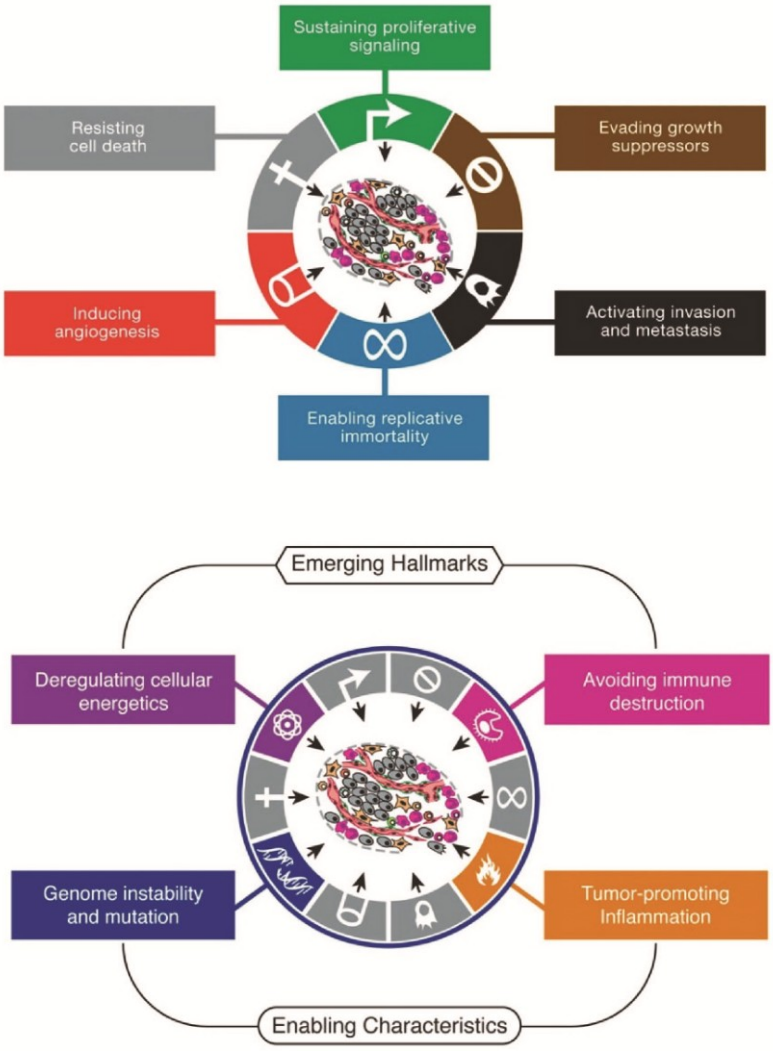

Figure 3 : Les « hallmarks » of cancer (d'après Hanahan and Weinberg, 2011).

La compréhension des interactions entre la tumeur et le microenvironnement est devenue l'un des enjeux fondamentaux de la recherche contre le cancer (Rapport ARC-INCa 2013).

Bien que les premières études montrant les effets des ligands sigma sur des lignées tumorales remontent aux années 90, le rôle propre de Sig1R dans la carcinogenèse reste peu exploré. L'utilisation de ligands sigma radio-marqués a démontré la présence de Sig1R dans différentes lignées cellulaires issues de tumeurs humaines et animales (Aydar et al., 2004; John et al., 1995; Vilner et al., 1995). Ce qui a été confirmé plus tard dans des lignées cellulaires issues de cancers mammaires, pulmonaires et prostatiques à l'aide d'anticorps dirigés contre Sig1R (Aydar et al., 2006). De la même façon, l'expression de Sig1R a aussi été observée par immunohistochimie dans des tissus cancéreux mammaires (Simony-Lafontaine et al., 2000; Wang et al., 2004). Ainsi, comparée à des tissus mammaires sains, l'expression de Sig1R est significativement plus élevée dans les tissus cancéreux et plus précisément dans des cancers progestérone-dépendant. Toutefois, dans ces études, la survie des patients ou la propension à développer des métastases n'ont pas été significativement corrélées à l'expression de Sig1R.

Dans des lignées cellulaires issues de différents cancers (sein, colon, leucémie lymphoïde, glioblastome), les ligands Sig1R réduisent la croissance et la viabilité *in vitro* mais aussi leur capacité à former des tumeurs *in vivo* (Achison et al., 2007; Azzariti et al., 2006; Spruce et al., 2004). Des agonistes de Sig1R ont également montré des effets inhibiteurs sur la prolifération, l'adhésion, la migration et la résistance à l'apoptose des cellules cancéreuses (Aydar et al., 2006; Kim et al., 2012; Megalizzi et al., 2007; Renaudo et al., 2007; Renaudo et al., 2004; Wang et al., 2004) mais peuvent aussi réduire l'effet inhibiteur des antagonistes de Sig1R (Spruce et al., 2004). *In vivo*, le 2-IPB (agoniste Sig1R et Sig2R) réduit la croissance de cellules cancéreuses pulmonaires humaines transplantées dans le flanc de souris immunodéficientes (Moody et al., 2000).

Par ailleurs, plusieurs études ont montré que l'invalidation de l'expression de la protéine Sig1R réduit l'adhésion et la résistance à l'apoptose des cellules cancéreuses, indépendamment de la présence d'un ligand sigma (Kim et al., 2012; Palmer et al., 2007; Renaudo et al., 2007). A l'inverse, en sur-exprimantsurexprimant la protéine Sig1R, on augmente la résistance à l'apoptose (Renaudo et al., 2007).

Dans le contexte du microenvironnement tumoral, la cocaïne, agoniste de Sig1R est impliquée dans la régulation de l'activité anti-tumorale des cellules immunitaires. En effet, elle favorise la sécrétion de cytokine anti-inflammatoire (IL-10) permettant une meilleure croissance de la tumeur qui échappe ainsi à sa destruction par le système immunitaire (Gardner et al., 2004; Zhu et al., 2003).

Au final, ces données suggèrent que Sig1R pourrait intervenir dans le phénotype et le développement tumoral. D'un point de vue diagnostique, des ligands sigma radioactifs ont été validés pour la détection de tumeurs de la glande pituitaire dans un modèle murin (Ramakrishnan et al., 2013). Il est ainsi envisageable de pouvoir utiliser Sig1R comme biomarqueur tumoral ou comme cible thérapeutique. Néanmoins, la fonction princeps de Sig1R dans la carcinogenèse reste à explorer.

J. Conclusion sur les fonctions physiopathologiques de Sig1R :

Dans cette partie, nous avons passé en revue les fonctions physiologiques et pathologiques de Sig1R. Il en ressort que cette protéine agit principalement à la manière d'un neuroprotecteur. Sa stimulation par des ligands permet d'éviter les dommages cérébraux induits par différentes pathologies (amnésies, AVC, dépression). A contrario, dans d'autres situations, l'activation de Sig1R par des agonistes peut exacerber des situations pathologiques (addiction, schizophrénie, infection par le VIH, douleur).

Il est intéressant de remarquer qu'à l'exception des ligands « addictifs » tels que la cocaïne ou la méthamphétamine, Sig1R et ses ligands ne présentent pas d'activité dans des conditions normales. Sa fonction ne se dévoile que lorsque la cellule se retrouve en situation de stress telle que l'ischémie (AVC), l'amnésie (chimique, physiologique ou pathologique) ou encore l'induction de stimuli nociceptifs. Cette fonction de Sig1R révélée dans des situations « stressantes » est en accord avec la démonstration par Hayashi et Su de la fonction de Sig1R en tant que protéine chaperonne sensible aux stress cellulaires (III.C) (Hayashi and Su, 2007).

Ainsi, la modulation de Sig1R par des ligands peut être une stratégie intéressante pour traiter certaines pathologies en activant ou inhibant Sig1R. Néanmoins, il convient d'évaluer les conséquences de tels traitements de sorte à ne pas créer les conditions

favorables à l'activation de Sig1R dans des processus néfastes (douleur ou addiction) en voulant activer sa fonction neuroprotectrice.

Dans les cancers, la surexpression de Sig1R semble promouvoir la maladie comme c'est le cas pour l'addiction. Alors que les antagonistes présentent un effet inhibiteur des fonctions pro-tumorales, on aurait dû s'attendre à ce que les agonistes présentent un effet potentiateur sur les fonctions pro-tumorales. Cependant, contre toute attente, les agonistes présentent majoritairement une fonction inhibitrice. Ainsi, le rôle de Sig1R dans les cancers semble être atypique et suggère une modulation de son activité différente de celle retrouvée dans le tissu cérébral.

De nouvelles études sont donc nécessaires afin de comprendre comment Sig1R intervient dans les cancers. Les premiers éléments de réponse obtenus par les travaux précédents de l'équipe indiquent que Sig1R régule l'activité ionique des cellules cancéreuses (Renaudo et al., 2007; Renaudo et al., 2004). D'autres travaux indiquent un rôle de Sig1R sur la composition lipidique de la membrane plasmique des cellules cancéreuses (Palmer et al., 2007), ou sur la régulation de l'activité mitochondriale (Achison et al., 2007; Spruce et al., 2004). Toutefois, les caractéristiques de cette modulation par Sig1R sont loin d'être pleinement comprises et nécessitent de nouvelles recherches.

III. Les fonctions de Sig1R dans la cellule

Dans la première partie, nous avons tenté de définir les rôles physiologiques de Sig1R à travers sa distribution et les effets induits par des molécules capables d'interagir avec cette protéine. Dans cette deuxième partie, nous allons changer d'échelle et nous intéresser à la structure de Sig1R et aux mécanismes moléculaires qui définissent ses fonctions au niveau cellulaire. Nous nous intéresserons à la fonction « chaperonne » de Sig1R et ses interactions privilégiées avec les protéines membranaires de la famille des canaux ioniques.

A. Structure de Sig1R

La purification du site de liaison sigma 1, le séquençage de la protéine Sig1R et le clonage du gène (SIGMAR1) remontent à 1996 (Hanner et al., 1996b). Sig1R est une petite protéine de 223 acides aminés (environ 25 kDa) qui ne présente pas d'homologie avec le reste des protéines de mammifères. La protéine connue la plus proche de Sig1R est une C7-C8 Stérol isomérase de levure qui possède seulement 30% de similitude avec Sig1R. Cependant, Sig1R ne présente pas d'activité stérol-isomérase (Hanner et al., 1996b).

Bien que la structure 3D de Sig1R ne soit pas encore disponible, plusieurs modèles issus d'analyses bio-informatiques, d'approches biochimiques et moléculaires (binding, mutagenèse dirigée) ont été proposés.

La séquence peptidique de Sig1R suggère la présence d'au moins un segment transmembranaire (Hanner et al., 1996b; Kekuda et al., 1996). L'analyse de la séquence, à l'aide de logiciels de modélisation indique cependant l'existence de deux domaines transmembranaires et un domaine hydrophobique, probablement en interaction avec la membrane lipidique (Aydar et al., 2002). Deux sites de liaison aux stérols (SBDLI (91-109) et SBDLII (176-194)) ainsi qu'un motif d'adressage dans le réticulum endoplasmique (RR) ont été par la suite identifiés (Pal et al., 2008; Sharma et al., 2010). Le site de liaison des ligands sigma sur Sig1R a été exploré par mutagenèse dirigée. Ainsi, les acides aminés A13, L28, A86, S98, Y103, L105, L106, I128, Y123 et ceux contenus entre les acides aminés R119 et G149 sont importants dans la liaison des ligands sigma (Brune et al., 2014; Ganapathy et al., 1999; Yamamoto et al., 1999).

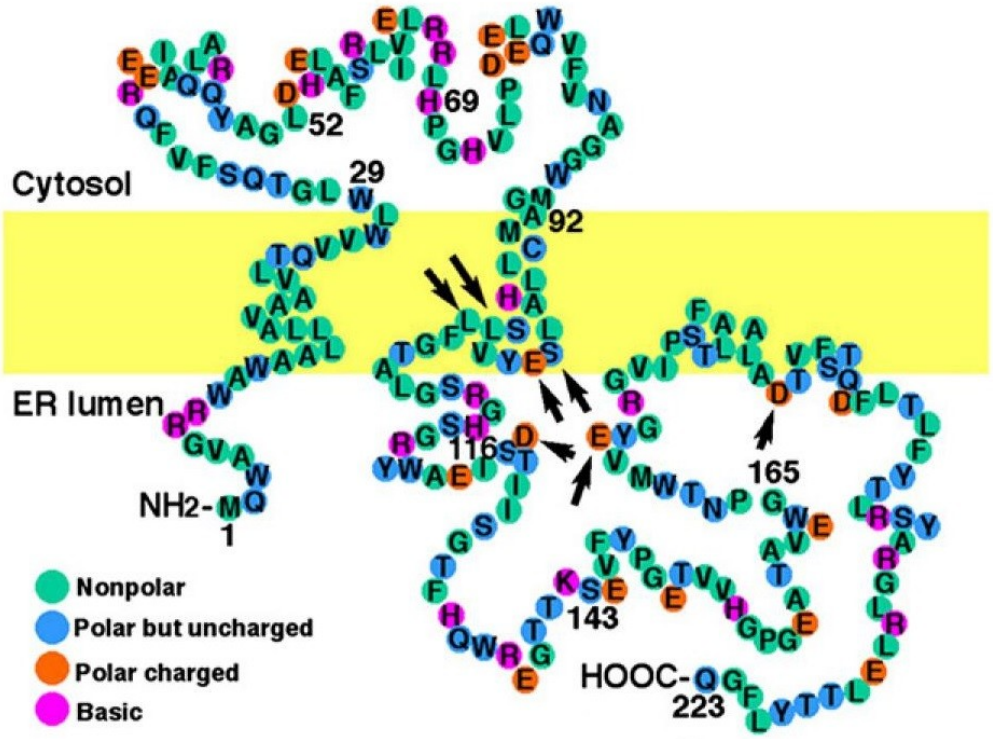

Figure 4 : Structure topologique théorique de Sig1R (d'après Hayashi et Su., 2007). Les flèches indiquent les acides aminés critiques pour la fixation des ligands.

Mais les acides aminés D126 et E172 sont, quant à eux, indispensables à cette liaison (Brune et al., 2014; Seth et al., 2001). Au niveau structural, les deux domaines de liaison des stérols (SBDLI et SBDLII) sont juxtaposés et permettent ainsi la formation du site de liaison des ligands (Pal et al., 2008). Avec l'identification de sa fonction en 2007, il a été remarqué que la partie C-terminale de Sig1R (102-223) interagissait avec le récepteur à l'IP3 (IP3-R) et la protéine BiP (GRP78) (Hayashi and Su, 2007; Ortega-Roldan et al., 2013; Wu and Bowen, 2008). Enfin, Sig1R possède plusieurs motifs de liaison pour le cholestérol dans sa partie C-terminale, un motif simple VEYGR et un motif contenant une superposition de deux motifs de liaison au cholestérol (LFYTLRSYAR) (Palmer et al., 2007), suggérant un rôle de Sig1R dans l'organisation de microdomaines lipidiques particuliers que l'on appelle « radeaux lipidiques » (III.D).

Le gène codant Sig1R (SIGMAR1), situé sur le locus p13.3 du chromosome 9, est composé de quatre exons pour une longueur totale de 3105 paires de bases. Au total, cinq variants d'épissage de ce gène ont été identifiés (Numéro d'accession Swiss prot : Q99720-1 ; Q99720-2 ; Q99720-3 ; Q99720-4 ; Q99720-5), mais seulement deux d'entre eux ont été étudiés physiologiquement. Le premier variant de la protéine Sig1R identifié est une forme

de Sig1R d'une masse de 19kDa pour laquelle il manque la partie codée par l'exon 3 (Q99720-3). Cette forme de Sig1R, détectée dans des cellules dérivées de leucémie lymphoïdes (cellules Jurkat), est incapable de fixer les ligands sigma (Ganapathy et al., 1999). Le second variant de Sig1R a été caractérisé plus récemment dans l'hippocampe de souris (Shioda et al., 2012). Dans cette étude, les auteurs ont identifiés un variant de Sig1R de 106 acides aminés pour lequel il manque la partie C-terminale de Sig1R (Q99720-4). Ils observent que cette forme courte de Sig1R dimérise avec la forme native de Sig1R. Par ailleurs, la forme courte de Sig1R est incapable de s'associer avec le récepteur à l'IP-3 et abolit l'influx calcique mitochondriale, mettant ainsi en avant le rôle de la partie C-terminale dans l'interaction avec l'IP3-R déjà observé par Wu & Bowen (Wu and Bowen, 2008). Ces résultats confirment les observations précédentes et le rôle de la partie C-terminale dans la fonction chaperonne de Sig1R.

Deux polymorphismes de Sig1R sont connus, G240T/C241T et A61C (entrainant la mutation Q2P) (Miyatake et al., 2004). Ces deux polymorphismes ont été étudiés avec plus ou moins de succès sur des cohortes de patients souffrant de la maladie d'Alzheimer, de schizophrénie ou de dépendance à l'alcool (II.A)(Feher et al., 2012; Huang et al., 2011; Maruszak et al., 2007; Miyatake et al., 2004; Uchida et al., 2005). Deux mutants ponctuels ont également été décrits (E102Q et c.672*51G>T dans la partie 3'UTR du gène codant pour SIGMAR1) dans des formes familiales d'ALS (II.G) (Al-Saif et al., 2011; Luty et al., 2010).

L'orientation de Sig1R dans les bicouches lipidiques a été soumise pendant plusieurs années à controverse. En effet, la première étude mentionnant la présence de deux segments transmembranaires précise que les extrémités N et C de la protéine sont cytoplasmiques (Aydar et al., 2002). Mais en 2007, Hayashi et Su décrivent une orientation opposée de Sig1R, les extrémités N et C terminales de Sig1R étant observées dans la lumière du réticulum endoplasmique (Hayashi and Su, 2007). Enfin récemment, Kourrich et al. ont observé que les extrémités N et C terminales sont orientées vers l'extérieur de la cellule, contredisant le modèle proposé par Aydar et confirmant celui proposé par Hayashi et Su (Kourrich et al., 2013).

Récemment, une modélisation 3D de Sig1R a été réalisée sur la base de la conservation des résidus importants au cours de l'évolution (Latha et al., 2013). Ainsi, les auteurs proposent une protéine avec sept segments hydrophobiques, mais ne précisent pas son arrangement et son orientation dans une bicouche lipidique. Ce modèle a récemment été utilisé pour élucider le site d'interaction des ligands sigma. Les auteurs observent que les

acides aminés E172, Y173 et I128 sont essentiels pour la fixation de la (+)-pentazocine (Brune et al., 2014). Ces données sont en accord avec les expériences précédentes (Ganapathy et al., 1999) et renforcent la validité du modèle *in silico* proposée.

En résumé, on dispose à présent d'un solide faisceau d'arguments pour postuler de l'organisation tridimensionnelle de Sig1R et des zones dédiées à ses différentes fonctions (protéine chaperonne, interaction avec les ligands, …). Cependant, la cristallisation de la protéine Sig1R insérée dans une bicouche lipidique permettra de vraiment conclure et proposer un modèle fiable et reconnu par tous.

B. Localisation de Sig1R dans la cellule

Avant de détailler la fonction cellulaire de Sig1R, il convient d'en présenter la distribution subcellulaire. Sig1R a été observé principalement dans le réticulum endoplasmique des cellules nerveuses et des oligodendrocytes (pour revue : (Hayashi and Su, 2005)). Dans le réticulum endoplasmique (RE), Sig1R est localisé dans des structures globulaires enrichies en certains lipides (galactosylcéramides, cholestérol, …) (Hayashi and Su, 2003, 2004b). Sig1R a également été observé de façon plus discrète sur l'enveloppe nucléaire, dans les mitochondries et à la membrane plasmique des cellules (Aydar et al., 2002; Hayashi and Su, 2007; Jiang et al., 2006; Klouz et al., 2002; Kourrich et al., 2013).

La localisation de Sig1R dans le RE apparaît toutefois hautement dynamique. En effet, le traitement par des ligands sigma ou la survenue d'un stress induisent la disparition de ces structures globulaires riches en lipides et une translocation de Sig1R vers la membrane plasmique (Hayashi and Su, 2003; Kourrich et al., 2013; Ruscher et al., 2011).

C. Rôle de Sig1R dans la réponse au stress cellulaire

La fonction primaire de Sig1R est longtemps restée une question ouverte. Il aura fallu 31 ans après sa découverte pour commencer à comprendre les mécanismes moléculaires associés à cette protéine. En 2007, Hayashi et Su, dans une étude qui fait date dans « l'histoire » de Sig1R, identifient Sig1R comme une chaperonne moléculaire activée par le stress du réticulum endoplasmique (Hayashi and Su, 2007).

Les protéines chaperonnes sont une classe particulière de protéines qui guident et assistent les autres protéines au cours de leur vie afin qu'elles effectuent la tâche pour laquelle elles sont dédiées. Une des familles de protéines chaperonnes les plus connues est la famille des protéines de choc thermique, ou HSP (Heat Shock Protein). Ces protéines, identifiées grâce à leur sensibilité à la chaleur, assistent les autres protéines au cours de leur biosynthèse au sein du réticulum endoplasmique jusqu'à leur adressage et leur activité dans le compartiment prévu.

La particularité de Sig1R est d'exercer sa fonction de chaperonne lors de stress cellulaires. Ainsi, Hayashi et Su observent que Sig1R est sensible au stress du réticulum endoplasmique. Le traitement des cellules avec la thapsigargine, connue pour bloquer la protéine SERCA (une pompe à calcium activée par l'ATP), induit une vidange du calcium stocké dans le RE. Une telle libération de calcium constitue un signal apoptotique. Mais la « vidange » des stocks calciques du RE entraine aussi une réduction de l'efficacité de la machinerie de « repliement » des protéines (Schroder, 2008). Les protéines synthétisées ne peuvent donc plus adopter leur conformation tridimensionnelle. Ces protéines non-repliées s'accumulent dans le réticulum endoplasmique et conduisent la cellule à initier une réponse particulière que l'on nomme « réponse aux protéines non-repliées » (ou UPR pour « Unfolded Protein Response »). Cette réponse repose principalement sur l'activation de trois protéines. Premièrement, ATF6 induit la synthèse de protéines chaperonnes permettant de faciliter le repliement des protéines accumulées dans le RE. Puis PERK et IRE1 arrêtent la traduction, augmentent la synthèse de chaperonnes mais aussi celles de protéines de la machinerie de dégradation associée au réticulum endoplasmique (ERAD) afin de pouvoir dégrader le surplus de protéines mal-repliées. Grâce à l'UPR, la cellule régule l'adressage des protéines dans le RE et évite l'accumulation de protéines non-fonctionnelles. Si l'UPR ne permet pas de résoudre le problème, alors l'apoptose sera initiée (Schroder and Kaufman, 2005).

L'initiateur de l'UPR est la protéine BiP (ou GRP78). Dans leur étude, Hayashi et Su démontrent que Sig1R et BiP sont associés en l'absence de stress. La libération du calcium du RE, suite à une situation de stress, entraine la dissociation de Sig1R et de BiP. La protéine BiP peut alors interagir avec PERK, IRE-1 et ATF6 dans un environnement riche en protéines non-repliées, et initie l'UPR. Dans le même temps, Sig1R s'associe au récepteur à l'IP-3 (IP-3R), un canal calcique activé par l'IP-3, et stabilise son activité à l'interface entre le RE et de la mitochondrie dans des domaines appelés MAM (Mitochondria-Associated

Membranes). Sig1R permet alors de soutenir les échanges calciques entre le RE et la mitochondrie et protège la cellule de l'apoptose (Hayashi and Su, 2007).

Les ligands sigma tels que la (+)-Pentazocine et le (+)-SKF-10047 miment l'effet du stress cellulaire en dissociant Sig1R de BiP. Les auteurs ont aussi remarqué que la protéine Sig1R, présente de façon constitutive une activité de foldase et peut participer au repliement des protéines, renforçant ainsi son implication dans l'UPR en tant que chaperonne. Récemment, la même équipe a montré que Sig1R s'associe à la protéine IRE-1 et régule sa stabilité et sa phosphorylation dans les domaines MAM, permettant alors une initiation plus rapide de l'UPR (Mori et al., 2013).

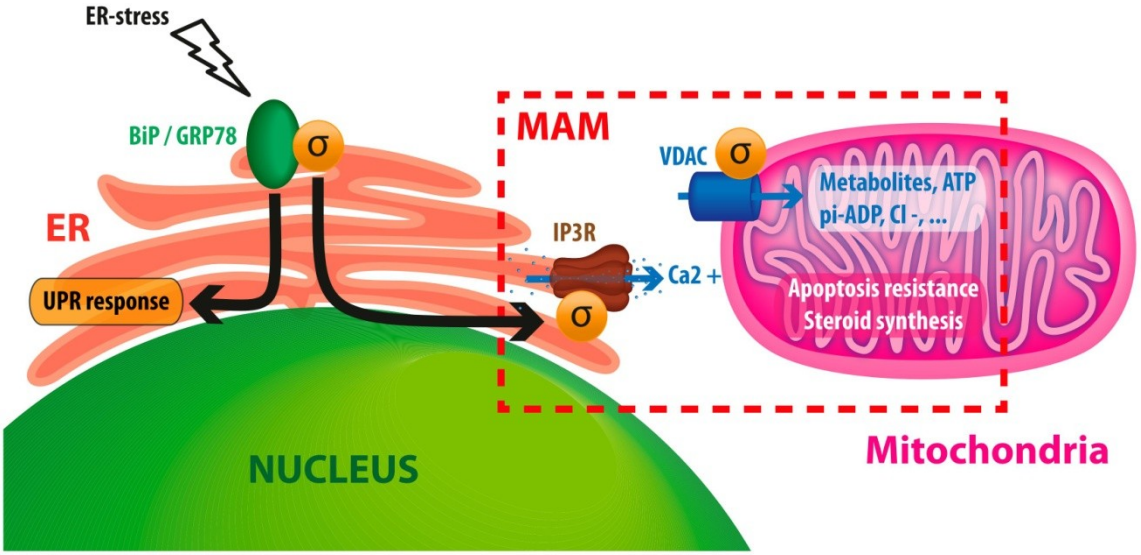

Figure 5 : Le rôle de Sig1R dans la réponse au stress cellulaires (adapté à partir de Hayashi and Su, 2007). UPR : réponse aux protéines non-repliées, ER : réticulum endoplasmique, IP3-R : Récepteur à l'IP3, VDAC : canal anionique sensible au voltage, MAM : domaines membranaires associés aux mitochondries.

L'implication de Sig1R dans la régulation du stress du RE a été confirmée dans d'autres modèles tels que les cellules cancéreuses et les cellules de la rétine (Ha et al., 2014; Schrock et al., 2013; Wang et al., 2012). Un article récent démontre que l'UPR régule l'expression de Sig1R à travers les protéines PERK et ATF4 (Mitsuda et al., 2011). Ceci suggère l'existence d'une boucle de rétrocontrôle positive où l'UPR favorise l'expression de Sig1R qui à son tour soutient l'UPR.

Cette fonction de chaperonne moléculaire, dépendante du stress cellulaire, est un modèle très intéressant dans la compréhension de la fonction primaire de Sig1R et de son implication dans les pathologies telles que la maladie d'Alzheimer, la maladie de Parkinson, l'AVC, l'ALS, le diabète ou le cancer (Duran-Aniotz et al., 2014; Hetz et al., 2013). Ainsi, manipuler l'activité de Sig1R par les ligands peut permettre de moduler le stress du RE et soigner certaines pathologies.

D. Sig1R et les lipides

Sig1R présente deux motifs de liaison au cholestérol. Une relation étroite existe donc entre Sig1R et les lipides. Confirmant les données structurales, Sig1R interagit avec le cholestérol et se distribue dans des zones riches de ce lipide à l'interface entre le RE et la mitochondrie (MAM) (Hayashi and Su, 2003, 2004b, 2005, 2010; Palmer et al., 2007). Dans les MAM, Sig1R ne se contente pas d'interagir avec le cholestérol mais organise sa compartimentalisation dans ces micro-domaines, créant des zones riches en cholestérol que l'on appelle « radeaux lipidiques » (Hayashi and Su, 2003).

En effet, la surexpression de Sig1R permet d'enrichir en cholestérol et gangliosides (GD1a) les radeaux lipidiques de la membrane plasmique, favorisant la neuritogenèse (Takebayashi et al., 2004). Lors d'occlusion ischémique du cerveau de souris, la stimulation de Sig1R par des agonistes, permet sa translocation à la membrane plasmique des astrocytes et des neurones, et l'enrichissement de la membrane en galactosylcéramide. Ceci permet d'établir des plateformes de signalisation essentielles à la formation de neurites des neurones (Ruscher et al., 2011).

Sig1R est donc en association étroite avec les lipides et module la composition des radeaux lipidiques. Ces radeaux lipidiques sont essentiels dans un grand nombre de processus tels que le transport vésiculaire, l'endocytose, la déformation de la membrane plasmique et la transduction de signaux moléculaires. Ainsi, moduler Sig1R pourrait permettre d'influer sur la composition et la formation de certains domaines lipidiques et donc d'agir sur différentes fonctions cellulaires.

E. Sig1R et les canaux ioniques

Les canaux ioniques sont des protéines responsables du maintien du potentiel de membrane des cellules et de l'excitabilité électrique (différences de potentiel électrique de part et d'autre des membranes lipidiques). Ces protéines forment des pores dans les membranes plasmiques et permettent la diffusion passive des ions selon leur gradient électrochimique. Elles génèrent ainsi un transport ionique excessivement rapide (10^7 à 10^8 ions par secondes), relativement sélectif (selon le canal ionique impliqué) et peu énergétique. L'ouverture et la fermeture de ces canaux ioniques sont finement régulés et peuvent dépendre d'une multitude de paramètres telles qu'un changement du potentiel de membrane (on parle alors de canaux ioniques voltage dépendants), une acidification extracellulaire, une augmentation de la concentration en calcium intracellulaire, la fixation de neurotransmetteurs ou d'ATP, etc….

L'existence des canaux ioniques a été postulée en 1952 par Hodgkin et Huxley pour expliquer le potentiel nerveux de l'axone géant de calamar (Hodgkin and Huxley, 1952). Cette hypothèse, qui leur vaudra le prix Nobel de Physiologie et Médecine en 1963, ne sera confirmée qu'en 1976 par la mesure directe de l'activité d'un canal ionique dans une fibre musculaire à l'aide de la technique de « patch-clamp » développée par Neher et Sakmann (Neher and Sakmann, 1976). Ces derniers obtiendront le prix Nobel de Physiologie et Médecine en 1982 pour cette découverte qui révolutionna le domaine des neurosciences et de la physiologie en général.

Depuis, les canaux ioniques ont été identifiés dans toutes les cellules excitables (neurones, cardiomyocytes, cellules musculaires, etc …) où ils interviennent dans la propagation des potentiels d'action (Hille, 1992). Mais ces transporteurs ioniques sont aussi présents dans les cellules dites « non-excitables » et participent aux transports transépithéliaux d'ions et d'eau, à l'origine de l'osmorégulation (Deutsch and Lee, 1988), aux phénomènes de sécrétion (neurotransmetteurs, bicarbonate, etc …) (Devaux et al., 1983; Rahamimoff et al., 1999), à l'activation des lymphocytes (Chandy et al., 1984; DeCoursey et al., 1984), etc …

Leur rôle fondamental dans cette multitude de processus physiologiques, font des canaux ioniques des acteurs importants dans l'apparition d'un certain nombre de pathologies que l'on nomme « canalopathies ». En effet, un défaut de conductances ioniques peut mener au développement de maladies telles que la mucoviscidose (CFTR), l'épilepsie (canaux

sodiques), les syndromes de QT long et de QT court (canaux sodiques et potassiques), le diabète (canaux potassiques) ou l'immunodéficience (canaux calciques) (Cant et al., 2014; Cregg et al., 2010; Feske, 2010; Kullmann and Waxman, 2010; Webster and Berul, 2013).

Les canaux ioniques sont connus depuis très longtemps pour être présents dans les cellules cancéreuses. La première étude qui mentionne la présence de courants ioniques dans des cellules tumorales date des années 60. Cette étude observait des courants sodiques et potassiques dans des sarcomes ascitiques d'Ehrlirch (Quastel, 1965).

Mais c'est l'identification formelle du canal potassique voltage-dépendant EAG-1 et la démonstration de son pouvoir oncogénique par Luis Pardo qui remit « au goût du jour » l'étude des canaux ioniques dans les cancers. En effet, l'expression de ce seul canal potassique voltage-dépendant est capable d'induire la transformation maligne de cellules normales (CHO) (Pardo et al., 1999). Depuis, un grand nombre de canaux ioniques ont été identifiés dans différents types de cancers et associés aux différentes « hallmarks » du cancer telles que la migration, l'invasion, la prolifération, la résistance à l'apoptose, l'angiogenèse tumorale ou la formation de métastases (Arcangeli, 2011; Arcangeli et al., 2009; Fiske et al., 2006; Fraser and Pardo, 2008; Kunzelmann, 2005; Le Guennec et al., 2007; Prevarskaya et al., 2010; Schonherr, 2005).

Canal ionique	**Expression dans les cancers**	**Rôle dans les « hallmarks »**
Canaux potassiques		
$K_v1.3$	?	Prolifération ?, Résistance à l'apoptose
$K_v1.5$	↑	Résistance à l'apoptose
$K_v10.1$ (EAG-1)	↑	Prolifération, Angiogenèse
$K_v10.2$ (EAG-2)	↑	Prolifération
$K_v11.1$ (hERG)	↑	Prolifération, Insensibilité aux signaux antiprolifératifs, Résistance à l'apoptose, Angiogenèse, Invasion et métastases
$K_{Ca}1.1$ (BKCa)	↑	Insensibilité aux signaux antiprolifératifs, Angiogenèse
$K_{Ca}2.3$ (SK3)	↑	Invasion et métastases
$K_{Ca}3.1$ (SK4, IKCa)	↑	Prolifération, Angiogenèse, Invasion et métastases
$K_{ir}3.1$ (GIRK1)	↑	Prolifération, Insensibilité aux signaux antiprolifératifs, Invasion et

		métastases
K_{2P}2.1 (TREK-1)	↑	Prolifération
K_{2P}9.1 (TASK-3)	↑	Prolifération, Insensibilité aux signaux antiprolifératifs, Résistance à l'apoptose
Canaux sodiques		
Na_v1.5	↑	Invasion et métastases
Na_v1.7	↑	Invasion et métastases
ASIC1	↑	Invasion et métastases
$E_{Na}C$	↑	Invasion et métastases
Canaux chlorures		
ClC-3	↑	Prolifération, Insensibilité aux signaux antiprolifératifs, Résistance à l'apoptose, Invasion et métastases
Canaux calciques		
Ca_v1.	↑	Prolifération
Ca_v2.3	↑	Prolifération
Ca_v3.	↑	Prolifération, Invasion et métastases
P2X5	↓	Insensibilité aux signaux antiprolifératifs
P2X7	↓	Résistance à l'apoptose
	↑	Invasion et métastases
ORAI (SOC)	↓	Résistance à l'apoptose
	↑	Angiogenèse, Invasion et métastases
TRPC1	↓	Insensibilité aux signaux antiprolifératifs
	↑	Angiogenèse
TRPC3	↑	Prolifération, Angiogenèse
TRPC4	↓	Insensibilité aux signaux antiprolifératifs
	↑	Angiogenèse
TRPC6	↑	Prolifération, Angiogenèse
TRPM2	↓	Résistance à l'apoptose
	↑	Angiogenèse
TRPM6	↑	Angiogenèse
TRPM7	↑	Prolifération, Angiogenèse
TRPM8	↑	Prolifération
TRPV1	↑	Invasion et métastases
TRPV6	↓	Résistance à l'apoptose
	↑	Prolifération

Tableau 2 : Les canaux ioniques dans les cancers (à partir de Prevaskaya et al., 2010).

La première étude relatant un rôle de Sig1R sur les canaux ioniques date de 1990. Dans cette étude, Kennedy et Henderson observent qu'un agoniste de Sig1R, le (+)-3-PPP, agit sur le potentiel de membrane, créant une dépolarisation liée à l'inhibition du courant potassique de type M dans les neurones sympathiques du ganglion hypogastrique (Kennedy and Henderson, 1990).

Il faudra cependant attendre quelques années pour que soit publiée la première étude observant l'effet d'un ligand sigma sur un courant ionique. En utilisant la technique de patch-clamp, Soriani et al ont démontré que la (+)-pentazocine inhibe plusieurs conductances potassiques dans les cellules mélanotropes de l'hypophyse de grenouille (Soriani et al., 1998).

1. Les canaux potassiques :

Depuis les travaux de Kennedy et de Soriani, différentes études, utilisant ou non des ligands sigma, ont mis en évidence plusieurs canaux potassiques comme étant des cibles de Sig1R.

Ainsi, les canaux potassiques voltage-dépendent $K_v1.4$, $K_v1.3$, $K_v1.5$, $K_v1.2$, $K_v11.1$, $K_v2.1$, le courant de type M (généré par les canaux $K_v7.1$ et $K_v7.2$) et le courant de type A (généré par les canaux $K_v4.1$; $K_v4.2$ et $K_v1.4$) sont modulés par Sig1R (Aydar et al., 2002; Crottes et al., 2011; He et al., 2012; Kinoshita et al., 2012; Kourrich et al., 2013; Lupardus et al., 2000; Renaudo et al., 2004; Soriani et al., 1999a; Soriani et al., 1999b; Soriani et al., 1998; Wilke et al., 1999; Zhang and Cuevas, 2005).

Il est intéressant de remarquer que Sig1R ne module pas toutes ces conductances de la même façon. En effet, les agonistes de Sig1R tendent à réduire l'amplitude des courants potassiques voltages-dépendants en diminuant la densité de courant. Cependant, le canal $K_v1.2$ semble échapper à cette règle puisque la cocaïne augmente l'amplitude du courant par son interaction avec Sig1R (Kourrich et al., 2013). Les ligands sigma modulent par ailleurs certaines caractéristiques biophysiques de ces canaux. Ainsi, les agonistes de Sig1R augmentent l'inactivation voltage-dépendante (état réversible dans lequel le canal ionique est ouvert mais non-conducteur) des canaux $K_v1.4$ et $K_v1.3$ et du courant de type M ($K_v7.1$ et $K_v7.2$) (Aydar et al., 2002; Kinoshita et al., 2012; Soriani et al., 1999b). Quelques différences persistent à propos de l'effet des ligands sigma sur l'inactivation du canal $K_v1.4$ puisque les travaux de Wilke et al. n'ont pas retrouvé l'effet observé par Aydar et Soriani (à

travers la mesure du courant de type A) (Aydar et al., 2002; Soriani et al., 1999b; Soriani et al., 1998; Wilke et al., 1999). Ces différences peuvent potentiellement s'expliquer par des différences de modèles d'études (lignée cellulaire (DMS-114) issue de cancer pulmonaire pour Wilke et al., expression hétérologue dans un ovocyte de xénope pour Aydar et al. et cellules mélanotropes de l'hypophyse de grenouille pour Soriani et al.

Les ligands sigma peuvent également agir sur l'activation (la probabilité d'ouverture en fonction d'un stimulus) du canal. Ainsi, Soriani et al. ont observé un effet de la (+)-pentazocine sur la sensibilité au voltage de l'activation du courant de type M. La (+)-pentazocine décale la courbe de dépendance au voltage de ce courant vers des potentiels plus dépolarisés, rendant le canal moins propice à s'ouvrir dans des conditions physiologiques (Soriani et al., 1999b), et à augmenter l'excitabilité des cellules du lobe intermédiaire de l'hypophyse de grenouille.

Pour expliquer les effets de Sig1R sur ces canaux potassiques, il a été proposé que Sig1R agisse de manière directe ou indirecte sur les canaux ioniques. Ainsi, Soriani proposa que Sig1R régule le courant de type A (K_v1.4) indirectement à travers les protéines G puisque leur inhibition abolit l'effet de la (+)-pentazocine sur les courants potassiques de type A enregistrés dans les cellules mélanotropes de l'hypophyse de grenouille (Soriani et al., 1998). En accord avec ce modèle, une étude récente a observé que la cyproheptadine, antagoniste des récepteurs sérotoninergiques ou histaminergiques, est capable de se lier à Sig1R et d'induire l'activation de petites protéines G favorisant ainsi l'activité du canal K_v2.1 (He et al., 2012).

Cependant, Lupardus et al. n'ont observé aucun effet de l'inhibition des protéines G sur les effets induits par les ligands sigma sur les courants potassiques dans les terminaisons nerveuses de l'hypophyse de rat (Lupardus et al., 2000). De plus, en utilisant la technique du patch excisé (dans laquelle un petit morceau de la membrane est isolé du reste de la cellule), les auteurs continuent d'observer l'effet de la (+)-pentazocine. Cela suggère que c'est l'interaction directe de Sig1R avec le canal ionique qui induit les effets des ligands sigma.

En 2002, Aydar et al. caractérisent une association directe entre Sig1R et le canal K_v1.4 exprimés dans l'ovocyte de xénope et proposent que Sig1R est une sous-unité auxiliaire Beta des canaux ioniques (Aydar et al., 2002). Depuis, il a été montré que Sig1R s'associe aux canaux K_v11.1, K_v1.3 et K_v1.2 (Crottes et al., 2011; Kinoshita et al., 2012;

Kourrich et al., 2013) renforçant l'idée que l'interaction directe de Sig1R avec le canal ionique explique son effet. Dans ce sens, Kinoshita et al. démontrent que l'effet de Sig1R sur l'inactivation du canal $K_v1.3$ est la conséquence de son interaction directe avec les domaines transmembranaires du canal ionique (Kinoshita et al., 2012).

Au cours de ma thèse, j'ai pu démontrer pour la première fois un mécanisme selon lequel Sig1R interagit avec un canal potassique et augmente son activité ionique en stimulant sa maturation et son expression à la membrane plasmique (Crottes et al., 2011)(V.A). Initialement mis en évidence pour le canal hERG ($K_v11.1$) dans des lignées leucémiques, ce mécanisme a depuis été confirmé pour le canal $K_v1.2$ dans le cerveau chez la souris (Kourrich et al., 2013).

Sig1R interagit donc de façon directe ou indirecte (via les protéines G) avec des canaux potassiques voltage-dépendants et module leur activité. L'hétérogénéité des effets de Sig1R observée (adressage à la membrane plasmique accrue, modulation des paramètres électrophysiologiques, régulation par les protéines G) sur ces canaux potassiques, rend difficile la proposition d'un mécanisme d'action unique. Ceci suggère que Sig1R pourrait réguler les canaux ioniques selon plusieurs mécanismes d'action en fonction du modèle étudié.

En plus des canaux potassiques voltage-dépendant, Sig1R régule aussi des canaux potassiques sensibles au calcium, à large et à petite conductance (BK et SK respectivement). Dans des neurones de l'hippocampe, l'activation de Sig1R par la (+)-pentazocine inhibe le courant SK et augmente la réponse calcique induite par le NMDA, facilitant ainsi l'apprentissage chez la souris (Martina et al., 2007). Néanmoins, une étude récente démontre que certains ligands sigma, utilisés à de fortes concentrations (DTG) sont capables d'inhiber les courants SK de façon indépendante de Sig1R (Lamy et al., 2010). Enfin, dans les neurones intracardiaques, Sig1R semble influer sur le courant potassique calcium-dépendant à forte conductance (BK) (Zhang and Cuevas, 2005). Aucun mécanisme moléculaire n'a été jusqu'alors proposé pour expliquer le lien fonctionnel entre Sig1R et ces canaux ioniques.

Au final, Sig1R est capable de moduler l'activité d'un grand nombre de canaux potassiques différents. Mais les caractéristiques de cette modulation sont encore mal comprises et suggèrent une hétérogénéité de l'action de Sig1R sur ces canaux potassiques.

2. Les canaux sodiques :

Sig1R contrôle également certains canaux sodiques et plus particulièrement sur un canal sodique voltage-dépendant, le canal $Na_v1.5$. Dans des cardiomyocytes et des modèles d'expression hétérologues (cellules HEK), plusieurs études ont observé que l'activation de Sig1R par un agoniste entraine une réduction du courant (Fontanilla et al., 2009; Johannessen et al., 2011; Johannessen et al., 2009). Etonnamment, les effets des agonistes de Sig1R sont inexistants dans des cardiomyocytes issus de souris Sig1R KO, mais la perte de l'expression de Sig1R n'affecte en rien l'amplitude du courant du canal $Na_v1.5$. Ceci suggère que les souris Sig1R KO ne nécessitent pas l'expression de Sig1R pour exprimer le canal $Na_v1.5$. Mais la présence de Sig1R peut permettre de moduler le canal $Na_v1.5$ de manière pharmacologique. En accord avec sa fonction de chaperonne activée par le stress cellulaire, on peut imaginer que Sig1R puisse intervenir dans la régulation du courant $Na_v1.5$ lors de situations de stress du tissu cardiaque (ischémie, etc …). Aucun résultat n'est encore survenu pour confirmer cette hypothèse.

Récemment, en collaboration avec l'équipe de M. Edwardson, j'ai pu mettre en évidence que Sig1R interagit directement avec le canal $Na_v1.5$ (Balasuriya et al., 2012). Nous observons ainsi que l'expression de Sig1R module l'activité du canal $Na_v1.5$ dans un modèle de cellules cancéreuses mammaires (MDA-MB-231) (0).

On pourra noter qu'une étude récente décrit un effet de l'agoniste de Sig1R, (+)-SKF-10047, sur les canaux sodiques voltage-dépendants $Na_v1.2$ et $Na_v1.4$ indépendamment de l'expression de Sig1R (Gao et al., 2012). Un même effet résiduel de cet agoniste a déjà été observé sur l'activité du canal $Na_v1.5$ en absence de l'expression de Sig1R (Johannessen et al., 2009).

3. Les canaux chlorures :

Les canaux chlorures sont longtemps restés peu étudiés car il était admis qu'ils n'intervenaient que de façon très anecdotique dans la régulation de l'excitabilité des cellules. Cependant, les découvertes, dans les neurones du canal chlorure activé par le GABA, important dans l'excitabilité neuronale, et dans les cellules épithéliales du canal CFTR impliqué dans la mucoviscidose, ont permis aux canaux chlorure de sortir de l'ombre (Duran et al., 2010).

Une seule étude rapporte un rôle de Sig1R sur l'activité d'un canal chlorure. Dans les cellules HEK293, les cellules de cancer du poumon de type SCLC et les cellules leucémiques de type T, la répression de l'expression de Sig1R - ou l'activation de Sig1R par l'Igmésine - inhibe le courant chlorure activé en réponse au changement de volume (Renaudo et al., 2007).

Cette étude issue de notre équipe, est la première à mentionner un effet de l'expression de Sig1R sur un canal ionique indépendamment de la présence de ligands sigma et donc à proposer Sig1R comme une protéine constitutivement « active ». Néanmoins, l'effet de l'expression de Sig1R se révèle lors du changement de volume. Ainsi en condition basale, Sig1R n'a aucun effet remarquable sur le courant chlorure. A l'instar de ce que nous venons de décrire pour les canaux sodiques, cette étude suggère que Sig1R n'intervient que dans des situations particulières, renforçant ainsi le concept de Sig1R en tant que protéine activée par un stress cellulaire, ici, un changement de volume dû à un choc osmotique.

4. Les canaux calciques :

Le calcium est l'un des seconds messagers les plus importants de la cellule. Contrôler les influx et efflux calciques dans l'espace et le temps est un enjeu essentiel pour la cellule et lui permet d'initier de nombreuses fonctions cellulaires (division cellulaire, apoptose, migration, etc …) (Parekh, 2011).

Depuis sa découverte, un nombre important de travaux ont décrit l'impact de Sig1R sur différentes conductances calciques. La première conductance calcique observée fut celle générée par le récepteur au glutamate de type NMDA, canal calcique impliqué notamment dans la mémoire et la plasticité neuronale.

L'expression de Sig1R module ainsi le courant généré par le récepteur au NMDA au cours du développement post-natal de l'hippocampe car il agit notamment sur le courant généré par le récepteur au NMDA (Sha et al., 2013). Plusieurs études ont montré un effet positif des ligands sigma sur ce courant calcique (Martina et al., 2007; Monnet et al., 1996; Monnet et al., 1992; Monnet et al., 2003; Smith et al., 2010; Zhang et al., 2012). Cependant d'autres études ont démontré un effet opposé de ces ligands sigma et donc une inhibition du courant calcique généré par le récepteur au NMDA (Herrera et al., 2008; Kume et al., 2002; Sershen et al., 1995; Zhang et al., 2011). Ainsi, on se retrouve face à une ambiguïté à

propos du rôle de Sig1R sur le récepteur NMDA. Pourquoi certains agonistes de Sig1R potentialisent leur activité alors que d'autres (et parfois les mêmes) l'inhibent selon les modèles ?

Dans les cellules nerveuses murines, Monnet et al. observent que les ligands sigma agissent sur le récepteur au NMDA en deux temps. Une première stimulation par le glutamate en présence des ligands sigma augmente l'influx calcique médié par les récepteurs au NMDA comparé à celui induit par le glutamate seul. Lors des stimulations suivantes, les ligands sigma inhibent l'influx calcique du récepteur au NMDA (Monnet et al., 2003). Ainsi, dans cette étude, les ligands sigma sont capables de favoriser mais aussi d'inhiber l'activité du récepteur au NMDA. Les auteurs proposent que cette désensibilisation du récepteur au NMDA pour le glutamate induite par les ligands sigma est la conséquence de l'activation de la voie de signalisation PLC-PKC par Sig1R à la membrane plasmique (Monnet et al., 2003), connue pour conduire à une désensibilisation de l'excitabilité neuronale de la douzième paire de nerfs crânien (Morin-Surun et al., 1999). Par ailleurs, les auteurs proposent que ce rôle de Sig1R sur le récepteur au NMDA s'ajoute à celui observé sur la régulation de l'homéostasie calcique du RE (Hayashi and Su, 2001).

Ainsi, l'activation de Sig1R pourrait donc moduler l'activité du récepteur au NMDA en fonction des voies de signalisation activées (notamment PLC-PKC) et de la concentration en calcium intracellulaire. Cela permet donc de supposer que l'activation ou l'inhibition du récepteur au NMDA induite par Sig1R est dépendante de la concentration en calcium intracellulaire et de l'état d'activation du récepteur au NMDA, expliquant les effets contradictoires observés dans ces études.

Dans ce sens, certains effets inhibiteurs des ligands sigma sur le récepteur au NMDA sont observés dans des conditions particulières d'ischémie ou d'activation des canaux sensibles au pH extracellulaire, ASIC1a (Herrera et al., 2008; Kume et al., 2002), connues pour conduire à d'importants influx calciques (Szydlowska and Tymianski, 2010).

Au niveau moléculaire, le récepteur NMDA est un tétramère composé de deux sous-unités NR1 et de deux sous-unités NR2. Sig1R interagit de façon directe avec le récepteur au NMDA en se fixant préférentiellement sur les sous-unités NR1 (Balasuriya et al., 2013) et module son activité en agissant sur la phosphorylation de ces sous-unités NR1 ou bien en modulant une voie de signalisation mettant en jeu des protéines G, la PKC et la PLC-gamma (Kim et al., 2008; Monnet et al., 2003; Zhang et al., 2011).

Sig1R module aussi l'activité de canaux calciques voltage-dépendants (Ca_v) de type L, N et P/Q. Contrairement au récepteur au NMDA, l'expression de Sig1R en elle-même ne semble pas affecter le courant généré par ces canaux Ca_v (Gonzalez et al., 2012). Majoritairement, les ligands sigma inhibent le courant calcique dépendant des Ca_v (Herrera et al., 2008; Lu et al., 2012a; Mueller et al., 2013; Tchedre et al., 2008; Zhang and Cuevas, 2002). Cependant deux études ont observé un effet positif de la (+)-pentazocine et de la prégnénolone sur ces canaux Ca_v (Sabeti et al., 2007; Soriani et al., 1999b). Il a également été mis en évidence que Sig1R interagit avec les canaux calciques voltage-dépendants de type L (Tchedre et al., 2008).

A la membrane du réticulum endoplasmique, il existe plusieurs canaux calciques nécessaires à la dissipation des réserves de calcium mais aussi aux échanges calciques avec les autres compartiments cellulaires tels que le cytoplasme, le noyau ou la mitochondrie. Leur activation entraine la libération de ces réserves et sert de signal à l'initiation de réponses cellulaires variées.

Sig1R est principalement localisé dans le réticulum endoplasmique. Hayashi et Su ont démontré que Sig1R intervenait dans la réponse au stress cellulaire en modulant l'activité du récepteur à l'IP-3, un canal calcique principalement localisé sur le réticulum endoplasmique (Hayashi et al., 2000; Hayashi and Su, 2001, 2007). Dans leur modèle, la stimulation des cellules par la (+)-pentazocine provoque la dissociation du complexe Sig1R/Bip (II-C) et la formation du complexe Sig1R/IP3-R. Sig1R stabilise la protéine IP3-R et soutient son activité permettant de conserver les échanges calciques entre le RE et la mitochondrie. D'autres ligands sigma ont montré un effet potentiateur sur sa fonction de transport d'ions calcium (Brent et al., 1996; Gasparre et al., 2012; Hayashi and Su, 2001, 2007; Ishima and Hashimoto, 2012; Tagashira et al., 2014; Wu and Bowen, 2008; Zhang et al., 2012).

Il a également été montré que Sig1R peut interagir avec les différentes isoformes de l'IP3-R (IP3-R1 et R3) (Abou-Lovergne et al., 2011; Hayashi and Su, 2001, 2007; Wu and Bowen, 2008).

Dans les hépatocytes, Sig1R s'associe à l'IP3-R1 mais ne participe pas à la libération de calcium induit par l'IP3. Cependant, Sig1R potentialise la synthèse de l'IP3 en réponse à la stimulation des récepteurs à la noradrénaline ou à la vasopressine. Les auteurs proposent que la faible expression de l'IP3-R1 dans ces cellules, et donc par conséquent sa faible

participation à la libération de calcium induite par l'IP3, peut expliquer l'absence d'effet de Sig1R sur ce paramètre (Abou-Lovergne et al., 2011).

Sig1R est donc capable de s'associer exclusivement à certains sous-types de récepteurs à l'IP3-R et d'en moduler l'activité. Cette interaction serait liée au domaine C-terminal de la protéine (Wu and Bowen, 2008), mais aucune hypothèse n'a été formulée pour expliquer la spécificité de Sig1R vis-à-vis de IP3-R1 et R3 par rapport à R2.

Concernant les RyR, une seule étude a démontré un effet de Sig1R sur ces récepteurs RyR. Contrairement à l'IP3-R, la (+)-pentazocine réduit l'activité du récepteur-canal RyR dans des cardiomyocytes (Tagashira et al., 2014).

5. Conclusion : Sig1R et les canaux ioniques

Les protéines chaperonnes sont connues pour interagir avec de nombreuses protéines et ont ainsi un degré de tolérance assez élevé pour la reconnaissance de leurs « clientes ». Sig1R, en tant que protéine chaperonne, ne fait pas exception à la règle et est capable de moduler une large variété de protéines et en particulier celles de la famille des canaux ioniques. Cependant, que ce soit l'expression de Sig1R ou l'utilisation de ligands sigma (agoniste ou antagoniste), on observe une grande hétérogénéité de leurs effets sur les canaux ioniques, rendant très difficile la suggestion d'un mécanisme d'action unique (Tableau 3). Toutefois, on peut observer une tendance des agonistes de Sig1R (Pentazocine, Igmésine, (+)-SKF-10047 …) à inhiber les courants potassiques, sodiques et chlorures. Dans le cas des courants calciques, on n'observe pas d'effet clair des ligands sigma.

Hayashi et Su ont décrit que les agonistes de Sig1R sont capables de dissocier Sig1R de la protéine BiP, mimant ainsi les conséquences d'un stress cellulaire (Hayashi and Su, 2007). Les antagonistes de Sig1R inhibent l'effet des agonistes. Dans leur modèle, lorsque Sig1R se dissocie de BiP, la protéine Sig1R interagit avec un canal calcique, l'IP3-R, et stabilise son expression et son activité ionique à l'interface de la mitochondrie et du réticulum endoplasmique. On devrait donc s'attendre à ce que les agonistes de Sig1R favorisent l'activité des canaux ioniques auxquels la protéine va s'associer. Or, cet effet n'est pas systématiquement observé. Au contraire, les agonistes de Sig1R ont tendance à inhiber l'activité des canaux potassiques, sodiques et chlorures et à avoir des effets divers sur l'activité des canaux calciques (y compris sur les récepteurs à l'IP3).

Cette hétérogénéité d'action suggère que le modèle d'action des ligands sigma proposé par Hayashi et Su est difficilement applicable à l'ensemble des canaux ioniques modulés par Sig1R. Il est donc possible que Sig1R puisse agir sur les canaux ioniques de différentes façons.

Canaux ioniques	**Conditions (ligands, shRNA, …)**	**Effets observés**	**Modèle d'étude**
		Canaux potassiques	
Kv1.2	Cocaine	↑ l'addressage du canal à la membrane plasmique	neurones (noyau accumbens)
	Sig1R KO	pas d'effet	
Kv1.3	Co-expression	↑ l'inactivation	ovocytes de xénopes
	Igm, Ptz	↓ la densité de courant	cellules cancéreuses
Kv1.4	Pentazocine	↓ la densité de courant ↑ l'inactivation	cellules mélanotropes de l'hypophyse de grenouilles
	(+)-SKF-10047	↓ la densité de courant ↑ l'inactivation	ovocytes de xénopes
	(+)-SKF-10047	↓ la densité de courant	neurones (hypophyse)
	(+)-SKF-10047	↓ la densité de courant	cellules cancéreuses
Kv1.5	(+)-SKF-10047	↓ la densité de courant	ovocytes de xénopes
Kv11.1	Igmésine	↓ la densité de courant	cellules cancéreuses
	shRNA	↓ l'addressage du canal à la membrane plasmique	
	Sur-expression	↑ la maturation et la stabilité du canal	HEK
	Co-expression	↑ la densité de courant	ovocytes de xénopes
Kv2.1	Cyproheptadine	↑ la densité de courant	
Kv7. (M-current)	Pentazocine	↓ la densité de courant ↓ l'inactivation ↓ l'activation	cellules mélanotropes de l'hypophyse de grenouilles
	DTG, Ptz	↓ la densité de courant	
BKCa	DTG, Ptz	↓ la densité de courant	neurones intracardiaques
SKCa	Pentazocine	↓ la densité de courant	neurones CA1 (hippocampe)
	DTG	↓ la densité de courant (indépendant de Sig1R)	neurones dopaminergiques

Canaux sodiques

Nav1.5	DMT	↓ la densité de courant	Cardiomyocytes
	Sig1R KO	Pas d'effet	
	Ptz, SKF	↓ la densité de courant	Cardiomyocytes, HEK
	Progestérone	Inhibe les effets des ligands sigma	
Nav1.2	(+)-SKF-10047	↓ la densité de courant (indépendant de Sig1R)	HEK
Nav1.4	(+)-SKF-10047	↓ la densité de courant (indépendant de Sig1R)	HEK

Canaux chlorures

VRCC	Igmésine	↓ la densité de courant	cellules cancéreuses
	Sur-expression	↑ la densité de courant	HEK

Canaux calciques

ASIC1a	PRE-084, Ibo	↓ la densité de courant	neurones (cortex)
Cav	Pentazocine	↑ la densité de courant	cellules mélanotropes de l'hypophyse de grenouilles
	Halopéridol	↓ la densité de courant	neurones intracardiaques
	Sig1R KO	pas d'effet	neurones
	PRE-084, Ptz	↓ la densité de courant (indépendant de Sig1R)	
	PRE-084, Ibo	↓ la densité de courant	neurones (cortex)
Cav1. (L-type)	(+)-SKF-10047	↓ la densité de courant	neurones (rétine)
	Pregnenolone	↑ la densité de courant	neurones (hippocampe)
	Ptz, SKF	↓ la densité de courant	neurones de la rétine
Cav2.1	(+)-SKF-10047	↓ la densité de courant	neurones (cortex)
Cav2.2	(+)-SKF-10047	↓ la densité de courant	neurones (cortex)
IP3-R	Methylphenidate	↑ la densité de courant	neurones (cortex pré-frontal
	PB-12	↓ la densité de courant	neuroblastome
	PB-12	↑ la densité de courant induite par le carbachol	neuroblastome
	Pentazocine	↑ la densité de courant induite par le carbachol	neuroblastome
	Ifenprodil	↑ la densité de courant	neurones (PC-12)
IP3-R1	Ptz, Igm, SKF	↓ la densité de courant	hépatocytes
IP3-R2	Pentazocine	↑ la densité de courant	cardiomyocytes

IP3-R3	siSig1R	↓ la densité de courant	CHO
Récepteur au NMDA	(+)-SKF-10047	↓ la densité de courant	neurones (rétine)
	MS-377, Hal	↓ la densité de courant	neurones dopaminergique
	Methamphétamine	↑ la densité de courant	neurones (hippocampe)
	Sig1R KO	↓ la densité de courant	neurones (hippocampe)
	Ibo, Ptz	↓ la densité de courant	Neurones (striatum)
	PRE-084, Ibo.	↓ la densité de courant	neurones (cortex)
	(+)-SKF-10047	↓ la densité de courant	neurones (cortex)
	Pentazocine	↑ la densité de courant	neurones CA1 (hippocampe)
	SKF, Ptz	↑ la densité de courant	neurones (hippocampe)
	Methylphenidate	↑ la densité de courant	neurones (cortex pré-frontal
RyR	Pentazocine	↓ la densité de courant	cardiomyocytes
TRPC5	BD-1047, IBP	↓ la densité de courant (indépendant de Sig1R)	HEK, cellules endothéliales
TRPM3	BD-1047, IBP	↓ la densité de courant (indépendant de Sig1R)	HEK, cellules endothéliales

Tableau 3 : Effets observés de Sig1R et de ces ligands sur les canaux ioniques. (DTG : Ditolylguanidine, DMT : N'N'-Dimethyltryptamine, Hal : Halopéridol, Igm : Igmésine, Ibo : Ibogaïne, KO : Knock-out, Ptz : Pentazocine, SKF : (+)-SKF-10047).

La synthèse des travaux réalisés sur la modulation des canaux ioniques par Sig1R semble indiquer que Sig1R peut agir directement ou indirectement avec certains canaux.

Sig1R peut influer directement, sur la stabilité, le trafic intracellulaire, l'expression à la membrane ou encore sur les paramètres biophysiques des canaux ioniques (II-E) (Crottes et al., 2011; Kinoshita et al., 2012; Kourrich et al., 2013; Kourrich et al., 2012; Soriani et al., 1999b; Su et al., 2010). Cependant les caractéristiques de son interaction avec ces transporteurs membranaires sont encore mal connues. Actuellement, Sig1R semble pouvoir interagir avec le domaine transmembranaire des canaux ioniques grâce à son extrémité C-terminale (Balasuriya et al., 2012; Balasuriya et al., 2013; Carnally et al., 2010; Kinoshita et al., 2012; Wu and Bowen, 2008).

Sig1R peut indirectement, agir sur les canaux ioniques en modulant des voies de signalisation (protéines G, PLC-PKC) ou en interagissant avec d'autres canaux ioniques (Herrera et al., 2008; Martina et al., 2007; Monnet et al., 2003; Soriani et al., 1999a; Soriani et al., 1998).

Toutefois, un certain nombre de points reste à élucider avant de pouvoir proposer un modèle qui décrit la relation entre Sig1R et les canaux ioniques.

F. Conclusion sur les fonctions moléculaires de Sig1R:

Durant cette partie, nous avons présenté la structure de Sig1R et sa fonction de protéine chaperonne active lors de stress cellulaire-. Il émerge ainsi un modèle dans lequel Sig1R pourrait se comporter comme une protéine chaperonne dont les canaux ioniques seraient les « clients privilégiés » (Su et al., 2010).

Cependant, l'hétérogénéité des interactions de Sig1R sur les canaux ioniques rend difficile la proposition d'un mécanisme d'action unique et nécessite de mieux comprendre la fonction de Sig1R vis-à-vis des canaux ioniques.

La relation qu'entretient Sig1R avec les lipides pourrait être un facteur contribuant à l'hétérogénéité de son action sur les canaux ioniques. La stimulation de Sig1R par des ligands exogènes permet la formation de radeaux lipidiques riches en cholestérol, gangliosides et galactosylcéramide (dans les cellules nerveuses) (III.D). Il est connu que la composition des radeaux lipidiques peut influencer les caractéristiques électrophysiologiques des canaux ioniques (Dart, 2010). Ainsi, il est possible que Sig1R puisse agir sur les canaux ioniques présents en modulant la composition de leur environnement lipidique.

Enfin, une situation de stress peut également être un facteur influant l'activité de Sig1R. Une déplétion des stocks calciques du RE, un choc thermique ou une privation en glucose (Hayashi and Su, 2007) (III.C) activent Sig1R. D'autres situations, telles que l'arrêt du repliement des protéines ou une perturbation de la biosynthèse lipidique, induisent un stress du RE et peuvent potentiellement activer la fonction chaperonne de Sig1R (Schroder, 2008). Il est donc possible que ces différents stimuli puissent moduler l'activité de Sig1R et induire différentes réponses de Sig1R sur les canaux ioniques.

Ainsi, Sig1R pourrait être une cible thérapeutique intéressante pour moduler l'excitabilité des cellules nerveuses et traiter certaines pathologies (dépression, Alzheimer, amnésies, AVC, etc …). Cependant des études sont encore nécessaires afin de cerner les paramètres du mécanisme d'action de Sig1R.

IV. Mon projet de thèse :

Bien que Sig1R ait été principalement étudié dans les SNC, plusieurs études suggèrent un rôle de la protéine dans les cancers (II.I). Ainsi, comprendre le rôle de Sig1R dans les cellules cancéreuses pourrait permettre de disposer d'une nouvelle cible thérapeutique intéressante. Son activité limitée aux tissus lésés serait par ailleurs un avantage et limiterait le risque d'effets secondaires des traitements anticancéreux ciblant alors l'activité de cette protéine.

Des travaux entrepris par mon équipe d'accueil ont démontré que Sig1R interagit avec des canaux ioniques exprimés dans les cellules tumorales. Ainsi, Sig1R module l'activité du canal potassique voltage-dépendant Kv1.3 et du courant chlorure VRCC dans des lignées cellulaires issues de cancers pulmonaires à petites cellules et de leucémies lymphoïdes (Renaudo et al., 2007; Renaudo et al., 2004). A travers cette interaction, Sig1R favorise la prolifération et la résistance à l'apoptose de ces cellules tumorales.

Ainsi, la fonction de Sig1R dans les cellules cancéreuses semble liée à son rôle sur l'activité des canaux ioniques.

Des travaux récents ont démontré l'implication de certains canaux ioniques dans les interactions qui se produisent entre les cellules cancéreuses et le microenvironnement tumoral. Le dialogue entre ces deux compartiments du tissu cancéreux est déterminant pour l'évolution de la maladie. La compréhension des mécanismes impliqués dans ce dialogue est donc un défi pour la mise au point de nouveaux traitements (Rapport ARC-INCa).

L'objectif de mon projet de thèse a été, dans un premier temps de caractériser la fonction moléculaire de Sig1R sur le canal ionique, hERG, connu pour son implication dans le dialogue tumeur / microenvironnement, (Arcangeli, 2011) dans les leucémies et les cancers colorectaux.

Dans un second temps, j'ai voulu évaluer le rôle de Sig1R, à travers son action sur ce canal ionique hERG, dans le dialogue tumeur / microenvironnement et ses conséquences sur le potentiel invasif des cellules cancéreuses.

Enfin, au cours de mon travail, j'ai été amené à explorer l'interaction entre Sig1R et le canal ionique Nav1.5 – également décrit comme impliqué dans le dialogue tumeur / microenvironnement (Brisson 2013) – et ses conséquences sur son activité ionique dans des cellules cancéreuses mammaires.

V. Résultats :

A. Sig1R régule le canal hERG dans les leucémies à travers un mécanisme post-transcriptionnel

1. Introduction :

Nous savons que Sig1R interagit avec plusieurs canaux potassiques voltage-dépendants (II-E). Les travaux antérieurs menés au laboratoire ont notamment montré que Sig1R intervient dans la prolifération des cellules de leucémie T à travers son interaction avec le canal potassique $K_v1.3$ (Renaudo et al., 2004).

Dans cette première partie de mon travail, j'ai posé l'hypothèse de l'existence d'une interaction entre Sig1R et hERG (human-Ether-a-go-go Related Gene), un autre membre de la famille des canaux potassiques voltage-dépendants ($K_v11.1$).

Le canal hERG est un canal principalement exprimé dans les cellules cardiaques, mais également dans le cerveau et l'hypophyse (Vandenberg et al., 2012). Dans le cœur, il intervient dans la phase de repolarisation du potentiel d'action et participe de ce fait à la régulation de la fréquence des battements cardiaques. Il se compose de l'assemblage de quatre sous-unités α qui forment le pore ionique.

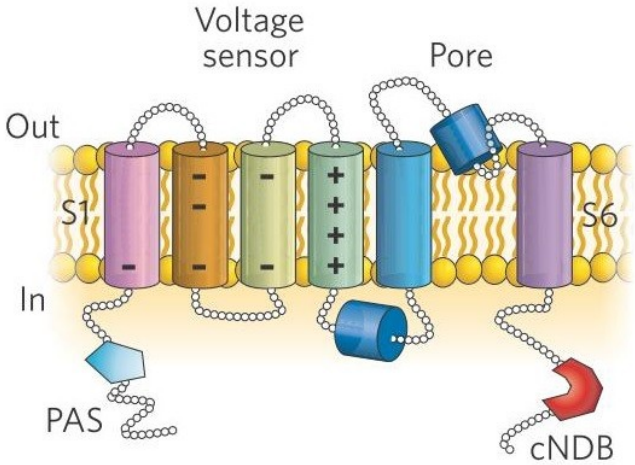

Figure 6 : Structure de la sous-unité α du canal hERG (d'après Sanguinetti and Tristani-Firouzi, 2006). (PAS : (Per-Arnt-Sim) domaine d'interaction avec les protéines, cNBD : domaine de liaison des nucléotides cycliques (AMPc)).

Chaque sous-unité α se compose de six segments transmembranaires, d'une boucle P (domaine pore) entre les segments 5 et 6, d'un domaine PAS (Per-Arnt-Sim) en partie N-terminale et d'un domaine de liaison au nucléotide (NBD) dans la partie C-terminale qui modulent respectivement les interactions avec les partenaires protéiques de hERG et avec l'AMPc. Les sous-unités α interagissent avec des sous-unités régulatrices β telles que Mirp1 ou Mink1 qui modifient les caractéristiques électrophysiologiques du canal. Des formes mutées de ce canal ont été identifiées dans des cas de syndrome de QT long (Sanguinetti and Tristani-Firouzi, 2006; Vandenberg et al., 2012).

Cependant, le canal hERG est aussi fréquemment observé dans de nombreux types de cancers tels que les leucémies myéloïdes chroniques ou aigües, les cancers colorectaux, gastriques, de l'endomètre, mammaires, prostatiques, ovariens, dans des glioblastomes ainsi que dans des lignées cellulaires issues de neuroblastomes et de cancer du poumon à petites cellules (Asher et al., 2011; Bianchi et al., 1998; Cavarra et al., 2007; Cherubini et al., 2000; Crociani et al., 2014a; Crociani et al., 2013b; Fiore et al., 2013; Glassmeier et al., 2012; Menendez et al., 2012; Pillozzi et al., 2002; Pillozzi et al., 2007; Pillozzi et al., 2011b).

Dans le contexte tumoral, hERG participe à la résistance des cellules à l'apoptose, au cycle cellulaire, à la résistance à la chimiothérapie, aux processus d'adhésion, de migration, d'invasion et d'angiogenèse (Asher et al., 2011; Crociani et al., 2003; Crociani et al., 2014a; Crociani et al., 2013b; Glassmeier et al., 2012; Jehle et al., 2011; Pillozzi et al., 2002; Pillozzi et al., 2007; Pillozzi et al., 2011b; Staudacher et al., 2014). L'une des fonctions remarquable de hERG est de participer au dialogue qui intervient entre les cellules cancéreuses avec le microenvironnement tumoral. En effet, les études menées par le groupe d'Annarosa Arcangeli démontrent que le canal hERG entretient une relation étroite avec la sous-unité β1 des intégrines (β1). L'activation de cette protéine par son ligand naturel, la fibronectine (composant essentiel de la matrice extracellulaire), induit le recrutement spatial du canal hERG au sein de microdomaines lipidiques membranaires. Dans ces radeaux riches en cholestérol, le canal s'associe à β1 mais aussi à la cavéoline-1 (protéine des cavéoles), à la protéine FAK (composante des adhésions focales) et à la protéine Rac-1 (associée à la réorganisation du cytosquelette lors de la migration cellulaire). L'activité de hERG dans ce macrocomplexe moléculaire permet de soutenir la phosphorylation de FAK et la fixation du GTP sur Rac-1 induites par la fibronectine, il en découle alors le renforcement de l'adhésion des cellules sur ce substrat (Cherubini et al., 2005).

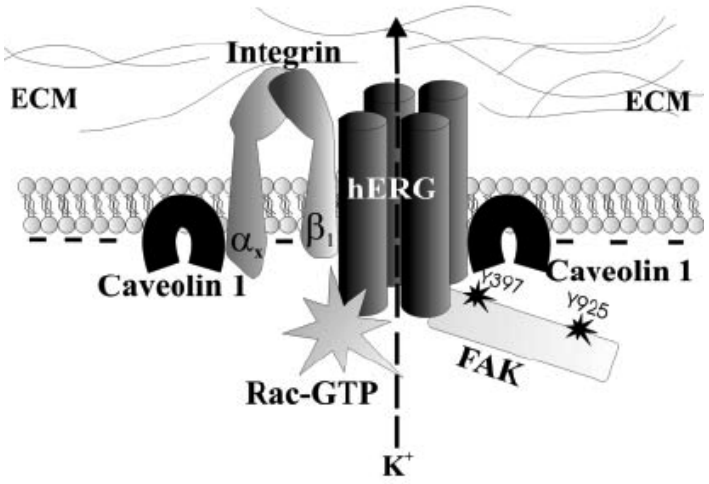

Figure 7 : Modèle de la régulation de la signalisation de la sous-unité β1 des intégrines par l'activité du canal hERG (d'après Cherubini et al., 2005). (ECM : matrice extracellulaire)

Dans des modèles de leucémies myéloïdes ou de leucémies lymphoïdes, la présence respective de VEGF ou SDF-1 dans le microenvironnement tumoral conduit au recrutement de leurs récepteurs (respectivement Flt-1 et CXCR4) dans le complexe hERG/ β1. Dans les leucémies lymphoïdes, l'activité de hERG renforce l'activation des voies de signalisation ILK, PI3K/Akt et ERK, induites par l'activation simultanée de β1 par la fibronectine et des récepteurs CXCR4 par la chimiokine SDF-1 sécrétée par les cellules mésenchymateuses de la moelle osseuse (Pillozzi et al., 2011b). Dans les leucémies myéloïdes, l'activité de hERG renforce l'activation des voies de signalisation PI3K/AKT et p38 induites par l'activation simultanée de β1 (par la fibronectine) et du récepteur Flt-1 (par le VEGF). L'une des conséquences de ce mécanisme est d'augmenter la sécrétion du facteur de croissance angiogénique, le VEGF, permettant ainsi d'entretenir une boucle autocrine (Pillozzi et al., 2007). Ainsi, hERG favorise la migration et l'invasion des cellules leucémiques myéloïdes, l'angiogenèse tumorale (induite par la sécrétion de VEGF) mais également l'extravasation des cellules dans la circulation sanguine (Pillozzi et al., 2007). Dans les leucémies lymphoïdes, hERG augmente la résistance des cellules à la chimiothérapie (Pillozzi et al., 2011b).

Cette relation étroite entre hERG et la sous-unité β1 des intégrines, a récemment été observée dans des cancers colorectaux (Crociani et al., 2013b). Tout comme dans les leucémies myéloïdes, hERG participe au soutien de la signalisation PI3K/AKT induite lors de l'activation de β1 par la fibronectine et contrôle la sécrétion du VEGF. hERG participe ainsi à

la progression des cancers colorectaux et au développement de métastases. Dans les glioblastome, hERG favorise également la sécrétion de VEGF (Masi et al., 2005).

J'ai dans un premier temps posé l'hypothèse d'une -interaction potentielle entre Sig1R et le canal hERG dans des cellules issues de leucémies myéloides. Le cas échéant, je me suis intéressé à l'identification des modalités de cette interaction ?

2. Article : Sig1R Protein Regulates hERG Channel Expression through a Post-translational Mechanism in Leukemic Cells

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Sig1R Protein Regulates hERG Channel Expression through a Post-translational Mechanism in Leukemic Cells*

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Sig1R (Sigma-1receptor) is a 25-kDa protein structurally unrelated to other mammalian proteins. Sig1R is present in brain, liver, and heart and is overexpressed in cancer cells. Studies using exogenous sigma ligands have shown that Sig1R interacts with a variety of ion channels, but its intrinsic function and mechanism of action remain unclear. The human ether-à-gogo related gene (*hERG*) encodes a cardiac channel that is also abnormally expressed in many primary human cancers, potentiating tumor progression through the modulation of extracellular matrix adhesive interactions. We show herein that sigma ligands inhibit hERG current density and cell adhesion to fibronectin in K562 myeloid leukemia cells. Heterologous expression in *Xenopus* oocytes demonstrates that Sig1R potentiates hERG current by stimulating channel subunit biosynthesis. Silencing Sig1R in leukemic K562 cells depresses hERG current density and cell adhesion to fibronectin by reducing hERG membrane expression. In K562 cells, *Sig1R* silencing does not modify hERG mRNA contents but reduces hERG mature form densities. In HEK cells expressing hERG and Sig1R, both proteins co-immunoprecipitate, demonstrating a physical association. Finally, Sig1R expression enhances both channel protein maturation and stability. Altogether, these results demonstrate for the first time that Sig1R controls ion channel expression through the regulation of subunit trafficking activity.

Sig1R (Sigma-1receptor) is a 25-kDa protein anchored to ER, mitochondria, nucleus, and plasma membranes (1–3). The structure is unrelated to other mammalian proteins (4) and presents two putative transmembrane segments (5). Sig1Rs are discretely distributed in the brain and peripheral tissues such as liver, kidney, heart, ovaries, and testis (2). Interestingly, Sig1Rs are overexpressed in numerous cancer cell types and interact with cell cycle and apoptosis pathways (6–8). The protein binds a large panel of exogenous compounds such as antipsychotics, opioids, and psychostimulants (2). It also interacts with endogenous steroids (9) and endogenous hallucinogenic tryptamines (10). However, in the absence of any characterized high affinity endogenous ligand, the classification of this protein as a classical receptor remains controversial. Sig1Rs participate in nociception, cardiac activity, memory, drug addiction, apoptosis, cell cycle, and immune response, but the primary molecular mechanism governed by Sig1Rs remains elusive (2). A recent breakthrough was introduced by Hayashi and Su (11), demonstrating that Sig1Rs physically associate with the chaperone Bip at the mitochondria-associated ER[4] membrane where they regulate calcium fluxes through inositol 1,4,5-triphosphate receptors. The emerging concept of the Sig1R as an interorganelle signaling modulator, activated either by ligands or cell stress, was then proposed (1). On the other hand, exogenous sigma ligands inhibit ion channels from different molecular families including voltage-dependent K^+ channels (Kv), voltage-dependent Ca^{2+} channels, voltage-dependent Na^+ channels, volume-regulated chloride channels, NMDA, or acid-sensing ion channels (1, 5, 7, 10, 12–15). Thus, Sig1Rs could exert its physiological role through the regulation of ion channels. Nevertheless, the constitutive activity of Sig1Rs on ion channels remains to be thoroughly addressed.

The involvement of abnormally expressed channels in the multiple facets of cancer development has been recently demonstrated (16, 17). We showed in this context that Sig1Rs modulate cell apoptosis resistance and cell cycle through the regulation of Kv1.3 and volume-regulated Cl^- channels in lung cancer and leukemia cells (7, 8). *hERG* encodes a voltage-dependent K^+ channel that regulates cardiac repolarization (18, 19). In a series of recent studies, the team of Arcangeli (20, 21) has proposed hERG as a biological marker of leukemia and several solid tumors. hERG forms membrane multi-protein signaling complexes with ECM receptors (integrins) and growth factor receptors (VEGF) to control adhesion, migration, differentiation, invasive process, and chemotherapy resistance of cancer cells. We investigate in the present study the putative links between hERG and Sig1R in a chronic myeloid cell line (K562), HEK 293 cells, and *Xenopus* oocytes. Using both electrophysiological and biochemical approaches, we demonstrate that the expression of Sig1R increases hERG current density through a regulation of channel subunit maturation and stability.

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[4] The abbreviations used are: ER, endoplasmic reticulum; Kv, voltage-dependent K^+ channels; FN, fibronectin; ECM, extracellular matrix; MOPS, 4-morpholinopropanesulfonic acid; Sig1R, Sigma1 receptor; hERG, human Ether-à-gogo-related gene.

MATERIALS AND METHODS

The K562 cell line was obtained from Dr. S. Brown (Cambridge, UK) and cultured in RPMI 1640 medium supplemented with 5% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. HEK 293 cells were cultured in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Chemicals and Reagents—Unless otherwise stated, all cell culture media, supplements, and antibiotics were purchased from Invitrogen. All other chemicals are from Sigma. Igmesine is a kind gift of Dr. F. Roman (Pfizer, Fresnes, France).

Animals—Female *Xenopus laevis* were anesthetized in 0.2% MS222 (tricaine methanesulfonate), according to the procedure recommended by our ethics committee. The surgery consisted in the removal of roughly five ovarian lobes containing oocytes. Following the surgery, the animals were kept in cold tap water to recover from anesthesia, monitored for 3 h, and finally replaced in their aquarium.

Preparation of cRNA—cRNAs were prepared from *hERG1* cDNA (kind gift of Dr. G. Robertson, Wisconsin University) or *Sig1R* cDNA, using a T7 or SP6 transcription kit (Ambion, Huntingdon, UK). cRNA concentration and integrity were estimated from a formamide/formaldehyde agarose gel in MOPS buffer.

Patch Clamp Experiments—K562 cells were prepared as described previously (7). The external solution was 45 mM KCl, 90 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes (pH adjusted to 7.4 with HCl, 285 mosm/liter). Soft glass patch electrodes (Brand, Wertheim, Germany) were made on a horizontal pipette puller (P-97; Sutter Instrument Co., Novato, CA) to achieve a final resistance ranging from 3 to 5 MΩ. The internal solution was 130 mM potassium aspartate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM Hepes, 2 mM ATP, and 100 μM GTP (pH adjusted to 7.2 with KOH, 290 mosm/liter). Electrical signals were amplified with an Axopatch 200B amplifier (Molecular Device, Foster City, CA) and acquired with a DIGIDATA 1440 interface and pCLAMP 10.2 software (Axon Instruments). K^+ currents were recorded at a 10-kHz sampling frequency and filtered at 2 kHz. Sigma ligands were added to external solutions that were administered in the vicinity of the cell under study through the use of a gravity-feed system (rate, 2 ml/min).

Double Electrode Voltage Clamp Experiments—*Xenopus* oocytes were maintained in modified Barth's saline medium adjusted to $[K^+] = 90$ mM (substituted for Na^+ to magnify K^+ driving force at −120 mV) for activation and in normal modified Barth's saline medium ($[K^+] = 1$ mM) for inactivation experiments. In the latter case, a three-pulse protocol was used: after a 1-s depolarizing step to 40 mV to fully activate hERG, 10-ms conditioning prepulses from 40 to −140 mV were applied, before repolarizing to +50 mV, where tail current values were recorded.

Adhesion Experiments—Fibronectin (FN) (Roche Applied Science) diluted to 40 μg/ml was coated overnight at room temperature onto a Nunc MaxiSorp flat-bottomed Immunoplate (Thermo Fisher, Langenselbold, Germany). The surface was then blocked with 40 mg/ml BSA for 1 h at room temperature. The plate was washed once with RPMI (without FBS). K562 cells were harvested and resuspended at 10^6 cells/ml in RPMI (without FBS). They were incubated fo r 1 h in the presence or absence of 10 μM E-4031 (a potent HERG channel blocker, Enzo Life Sciences, Lausen, Switzerland) or 10 μM igmesine. 10^5 cells were then plated in each well, and the plate was incubated fo r 2 h at 37 °C, 5% CO_2. Medium and unbound cells were removed by three PBS^+ washes. 0.2% (w/v) crystal violet, 20% (v/v) methanol solution was added and incubated for 20 min. The plate was washed thrice by immersion in water and dried before the addition of lysis buffer (75 mM NaCl, 25 mM Tris, pH 7.4, 10% SDS). Lysis was conducted under intermittent agitation for 20 min before reading absorbance at 540 nm on an iEMS plate reader (Labsystems, Helsinki, Finland).

Western Blot Experiments—Oocytes were homogenized in 20 μl/oocyte of 250 mM sucrose, 20 mM Tris-HCl, pH 7.4, containing 0.5 mM Pefabloc® (Roche Applied Science) and kept on ice at all times. The homogenate was prepared as described previously (22). The proteins were blotted onto PVDF (Millipore, Molsheim, France) membranes, using a Biometra semi-dry transfer system (Whatman, Göttingen, Germany). hERG was detected in the samples using a 1:2500 dilution of a polyclonal anti-hERG1(CT) pan antibody (Alexis, San Diego, CA) and a peroxidase coupled-anti-rabbit IgG secondary antibody (1:80000 dilution). Sig1R was detected using a 1:1000 dilution of a polyclonal Sigma receptor antibody from Santa Cruz Biotechnology (Heidelberg, Germany) and anti-goat IgG secondary antibody (1:40000). Both signals were visualized using ECL reagent (Pierce) on a Fusion FX-7 image acquisition system (Vilber Lourmat, Torcy, France). For K562 cells, total membrane proteins were isolated as follows: the cells were briefly washed with cold PBS, and then cell lysis was done in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) containing 0.5 mM Pefabloc for 15 min on ice. The homogenate was centrifuged successively at 350 × *g*, 1,400 × *g*, and 3,000 × *g* for 10 min at 4 °C, and the pellet was discarded after each centrifugation. The final supernatant was ultracentrifuged at 255,000 × *g* for 2 h at 4 °C. The protein pellet was solubilized in a suitable volume of lysis buffer, and the proteins were assayed as previously mentioned. Sig1R was detected as described above. For control of loading, we used either anti-actin (1:2000), anti-α-tubulin (1:50,000), or anti-calnexin (1/1000) antibodies (Sigma). Densitometric analysis of the data were performed with Image J analysis software (National Institutes of Health), and the results were corrected for protein loading by normalization for α-tubulin, actin, or calnexin expression.

shRNA Transduction—Lentiviral particles were obtained from Sigma (MISSION® shRNA lentiviral transduction particles). On day 1, K562 cells were plated in a 6-well plate at a density of 20,000 cells/well in complete medium. On day 2, medium was removed, and cells were incubated in complete medium containing 8 μg/ml of hexadimethrin bromide (Aldrich) and transduced at a multiplicity of infection of 5. Clones SHC002V (nontarget shRNA) and SHVRC-TRCN0000061011 (shSig1R targeted) were used for transduction. On day 3, a new transduction round was applied. On

day 6, puromycin (0.5 mg/ml) was added in fresh medium to start selection of transduced cells.

Co-immunoprecipitation—On day 1, cell culture dishes were placed on ice and washed three times with ice-cold PBS^+ (PBS with 0.5 mM Ca^{2+}). Then ice-cold lysis buffer (150 mM NaCl, 0.5 mM EDTA, 50 mM Tris-HCl, pH 8.00, 1% dodecyl-β-D-maltoside) was added (1 ml for two 100-mm dishes). For experiments using Sig1R ligand, the cells were incubated for 30 min with 10−5 M igmesine prior to lysis. The cells were scraped off, and tubes were placed fo r 2 h on a low speed rotator at 4 °C. In the meantime, anti-mouse IgG (whole molecule)-agarose beads were incubated in PBS (2% BSA). Then cell lysate was cleared by 10 min of centrifugation at 13,000 rpm. Supernatant was placed in a fresh tube kept on ice, and total protein concentration was determined. 6 mg were used for each sample and incubated in 2 ml (final volume) of 150 mM NaCl, 0.5 mM EDTA, 50 mM Tris-HCl, pH 8.00, and 0.5% dodecyl-β-D-maltoside. Anti-Myc tag IgG (1/500) (Euromedex, France) was added to the suspension and incubated at 4 °C fo r 1 h on the low speed rotator. 20μl of saturated beads were then added to each 2-ml sample and agitated by rotation at 4 °C overnight. Next day, the tubes were centrifuged, and the supernatants were removed. The beads were washed in PBS with 0.5% DMM for 5 min and then washed four times for 5 min in PBS with 0.1% DMM. Finally, the beads were resuspended in 20 μl of 2× loading buffer. The samples were heated at 95 °C for 3 min and were run on a 7–12% SDS-PAGE. hERG was probed with 1:2500 dilution of a polyclonal anti-hERG1(CT) pan antibody as described above. Sig1R was probed with 1:1000 dilution of a rabbit anti-Sig1R polyclonal antibody (Santa Cruz, Heidelberg, Germany) and detected with an anti-rabbit IgG-peroxidase (1:80000 dilution). The signal was visualized as described above.

Flow Cytometry—K562 cells were incubated for 20 min at 4 °C in a solution of PBS, 3% FBS, 2 mM EDTA containing an rabbit antibody directed against an external loop of hERG (Alomone Labs, Jerusalem, Israel). After washing in PBS, 3% FBS, 2 mM EDTA, the cells were stained for 20 min at 4 °C with Alexa-Fluor 488 conjugated anti-rabbit IgG (Santa Cruz Biotechnologies). The cells were analyzed with a FASCalibur flow cytometer and Cell Quest pro software (Becton Dickinson, Bedford, MA). The use of an appropriate isotypic control allowed eliminating nonspecific signal in the population.

hERG-transduced HEK 293—The coding sequence of *hERG1* was subcloned into the mammalian expression vector pPRIhygro to generate pPRIhygro-*hERG1*. pPRIhygro is derived from pPRIpu (23), where puromycin resistance gene was exchanged for hygromycin resistance gene (sequences of pPRIpu and pPRIhygro are available on request). Highly pure recombinant plasmids were obtained by anion exchange chromatography (NucleobondAx, Macherey-Nagel, Dären, Germany) and were used to stably transduce HEK 293 cells. For transduction experiments, HEK293 cells were seeded at 30–40% density in 100-mm dishes in DMEM supplemented with 10% FCS. To generate retroviruses, 293T cells were transfected the following day with 10 μg of an empty pPRIhygro plasmid or the pPRIhygro-*hERG1* construct and with 5 μg of pCMV-VSVG and 5 μg of pCMV-gag-pol plasmids using the classic calcium phosphate transfection technique. 6 h after transfection, the cells were washed, and fresh medium was added. Replication-defective retroviruses were recovered in the culture medium between 24 and 72 h post-transfection. These retroviral supernatants were filtered through sterile 0.45-μm filters and then added directly to HEK 293 cells in the presence of 4 μg/ml hexadimethrin bromide to enhance retroviral transduction efficiency. On day 6, hygromycin (100 ng/ml) was added in fresh medium to start selection of transduced cells. Western analyses were performed to check correct expression of hERG1.

HERG/cmycSig1R-transfected HEK 293—Similarly, *cmyc-Sig1R* cDNA (c-Myc tag added in phase at the N-terminal part of the protein) (7) was subcloned in pPRIPu vector. 10 μg of *pPRIPu-cmyc-Sig1R* cDNA was transfected in HEK stably expressing hERG1 using the classic calcium phosphate transfection technique. As control, a cmyc-eGFP cDNA sequence was subcloned in pPRIPu vector and transfected in the same way (all of the cDNA sequences are available on request). Obtention of stable HEK cells expressing hERG/cmycSig1R and hERG/cmyc-eGFP was achieved by puromycin selection.

Real Time PCR—Measurements were performed with a Light Cycler 1.5 (Roche Applied Science) using SYBR green I dye detection according to the manufacturer's recommendations. cDNA, synthesized from 4 μg of total RNA using random primers and Superscript III (Invitrogen), was added to a reaction mixture (Faststart DNA SYBR green I; Roche Applied Science) with appropriate primers at 0.5 μM each. The relative mRNA abundance was calculated using a standard curve method. Expression levels were normalized to the levels of the constitutively expressed 36B4 ribosomal protein mRNA. Oligonucleotides used for PCR were: *hERG1*up, 5′-TGAGGGCATTAGCTGGTCTAACT-3′; *hERG1*dw, 5′-GCAGTAAATAGCAGAAAAGTCCTTGA-3′; h36B4up, 5′-AATCCCTGACGCACCGCCGTGATG-3′; and h36B4dw, 5′-TGGGTTGTTTTCCAGGTGCCCTCG-3′.

Pulse-Chase Experiments—To analyze hERG maturation, HEK293 cells expressing GFP or sigma1R and hERG grown in 6-cm diameter dishes were incubated for 1 h in methionine/cysteine-free DMEM, then incubated for 10 min in the presence of 100 μCi [^{35}S]methionine (PerkinElmer Life Sciences, Waltham, MA), and chased for increasing amounts of time (up to 3 h) in the presence of DMEM containing 10% FBS as described previously (24). The cells were lysed in radioimmune precipitation assay buffer containing protease inhibitors and the resulting protein G-Sepharose clarified lysate immunoprecipitated with anti-hERG antibodies (Enzo Life Sciences, mAb A12) fo r 3 h on ice. Protein G-Sepharose beads were then added for 45 min. After five washes, immunoprecipitated material was resolved by SDS-PAGE. Immunoprecipitated hERG was then visualized by fluorography on x-ray films. To monitor hERG stability, a similar experimental approach was undertaken except that the pulse with [^{35}S]methionine was for 1 h, and the chase lasted up to 8 h. Biological replicates were performed in duplicate, and technical replicates were in triplicate. Quantitation was performed by scanning densitometry.

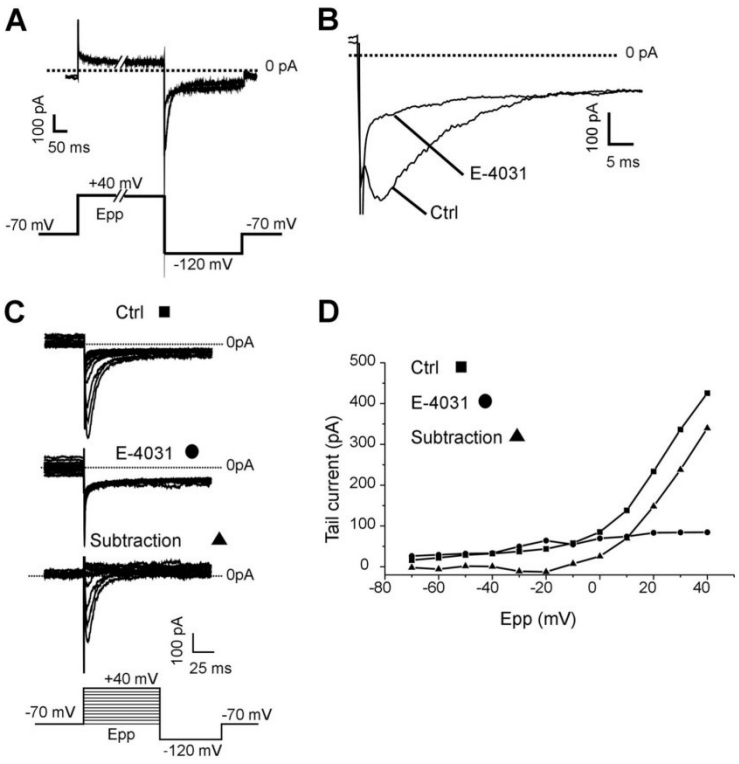

FIGURE 1. **Characterization of hERG currents in K562 cells.** *A*, superimposed tail currents recorded at −120 mV following a 2-s prepulse at +40 mV (*Epp*) to fully activate hERG channels, in the absence (control) or the presence of E4031 (1 μM). *B*, detail of the tail currents presented in *A*. *C*, families of tail currents recorded following prepulses from −70 to 40 mV in the absence (control) or the presence of E-4031 (1 μM). The *bottom current traces* represent the graphic subtraction. *D*, corresponding I/V plots. Tail current amplitudes are plotted against prepulse potential (*Epp*). *Ctrl*, control.

RESULTS

Sigma Ligands Inhibit hERG Current and Adhesion to FN in K562 Cells—Patch clamp experiments were performed in the whole cell variant of the whole cell technique. In high extracellular K^+ concentration, fast repolarizations at −120 mV following a 2-s prepulse at 40 mV gave rise to rapidly inactivating inward tail currents, fully abrogated by perfusion of the specific hERG inhibitor E-4031 (1 μM) (25) (Fig. 1, *A* and *B*). Amplitude of tail currents was dependent on prepulse amplitude, and graphical subtraction revealed that E-4031 inhibited a voltage-dependent conductance with an activation threshold of approximately −20 mV (Fig. 1, *C* and *D*). Altogether these data confirmed the presence of functional hERG channels in our K562 cell line (26). To verify a potential interaction between the Sig1R and hERG, we studied the effects of sigma-1 selective ligands, *i.e.* igmesine and (+)pentazocine (4, 7, 14, 27) on tail currents. Extracellular applications of igmesine or (+)pentazocine reversibly depressed the tail currents recorded at −120 mV and following an activating prepulse at 40 mV. The current was reduced by 40.85 ± 2.83% (n = 10) and 21.19 ± 1.77% (n = 3) for igmesine and (+)pentazocine, respectively (10 μM each). The maximal inhibition occurred within 3 min following the onset of drug applications (Fig. 2*A*). The effect of igmesine (10 μM) was next studied on hERG I/V plots recorded from −70 to 40 mV. Igmesine (10 μM) produced a dramatic reduction of the maximal current amplitude for potentials ranging from −10 to 40 mV (Fig. 2, *B* and *C*), suggesting that the drug mainly affected current density. Fitting steady-state activation plots using a Boltzmann function revealed a nonsignificant 4-mV leftward shift in voltage dependence (−0.47 ± 0.89 mV (n = 14) and −4.4 ± 1.76 mV (n = 10)) in the absence or presence of igmesine, respectively, NS, Mann-Whitney). Igmesine changed neither the fast nor the slow deactivating components of hERG tail current recorded at −120 mV (Fig. 2*D*). Taken together, these results suggest that Sig1R is functionally linked to hERG channels. We then explored the role of this link on the integrin-dependent cell adhesion to ECM *in vitro*. K562 cells express a single subtype of FN-selective integrin, *i.e.* the $\alpha_5\beta_1$ (28). Both E-4031 and igmesine inhibited specific FN-dependent cell adhesion (Fig. 2*E*). Interestingly, the effects of igmesine on FN adhesion were not additive with those produced by E-4031 (Fig. 2*E*), strongly suggesting that Sig1Rs modulate, at least in part, FN adhesion through the control of hERG.

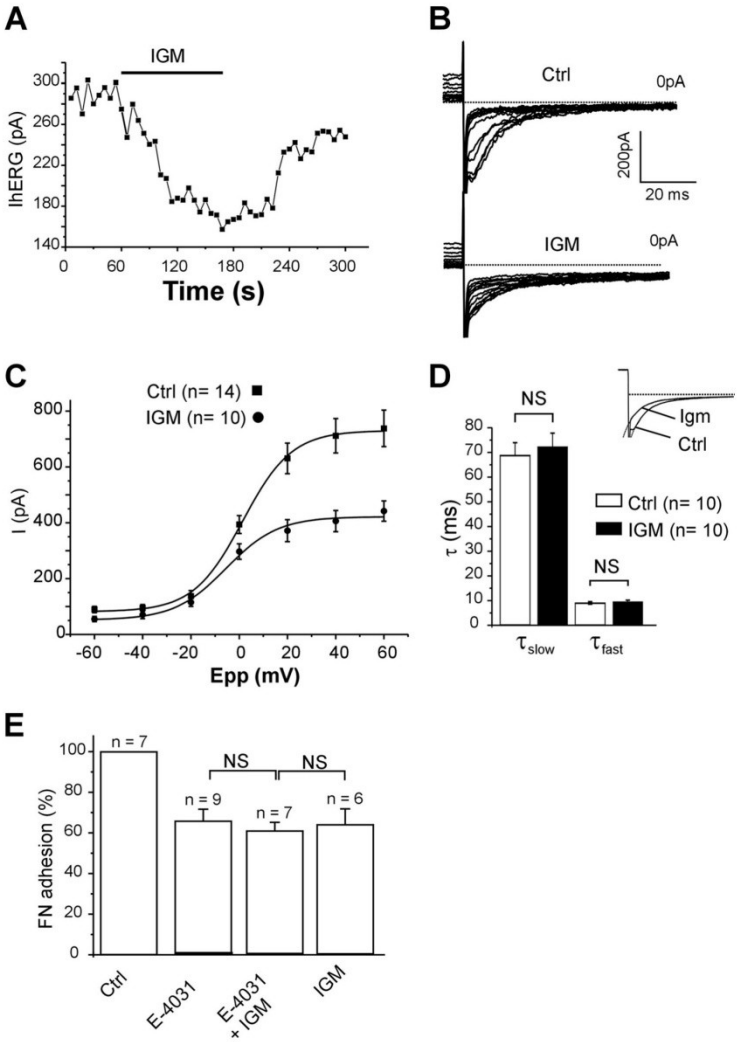

FIGURE 2. **Sigma ligands depress hERG current and cell adhesion to FN in K562 cells.** *A*, time course of hERG current recorded at −120 mV after a 40-mV prepulse in a K562 cell. Igmesine (*IGM*, *left panel*, 10 μM) was applied to the external side of the cells during the lap of time represented by the *black bar*. *B*, families of hERG currents recorded at −120 mV following prepulses from −70 to 40 mV in a single cell in the absence (upper panel, control) or the presence of igmesine (10 μM, *lower panel*, *IGM*). *C*, corresponding I/V plots. Tail current amplitudes are represented as a function of prepulse potential (*Epp*) and fitted with a Boltzmann function. *D*, histogram showing slow and fast deactivating constants at −120 mV in the absence (control) or the presence of igmesine (*IGM*, 10 μM). Deactivation was fitted with a double-exponential function. The *inset* shows representative superimposed tail currents recorded before and during igmesine (*Igm*) application. The values are the means ± S.E. *NS*, not significant (Student's *t* test). *E*, histogram representing the percentage of K562 cells adhering to FN, in control conditions (100%), in the presence of E-4031 (10 μM), E4031 + igmesine (10 μM each), or igmesine alone (10 μM). The values are the means ± S.E. of six to nine independent experiments. *NS*, not significant (Student's *t* test). *Ctrl*, control.

Sig1R Expression Potentiates hERG Current Density in Xenopus Oocytes—To address the function of Sig1R on hERG in the absence of any exogenous ligand, we next studied the effects of Sig1R expression on hERG currents expressed in *Xenopus* oocytes. Injection of *hERG* cRNA gave rise to voltage-dependent tail currents that were absent in water-injected oocytes (Fig. 3*A*, *left* and *middle panels*). Co-injection of *Sig1R* cRNA with *hERG* cRNA resulted in a 5-fold increase in current amplitude (Fig. 3, *A* and *B*). Sig1R expression did not significantly affect voltage-dependent activation (Fig. 3*C* and Table 1) or inactivation parameters (Fig. 3*D* and Table 1), indicating that Sig1Rs mainly modulate hERG current density. To further explore this hypothesis, Western blot analysis from control and injected oocytes were performed using the same cRNA concen-

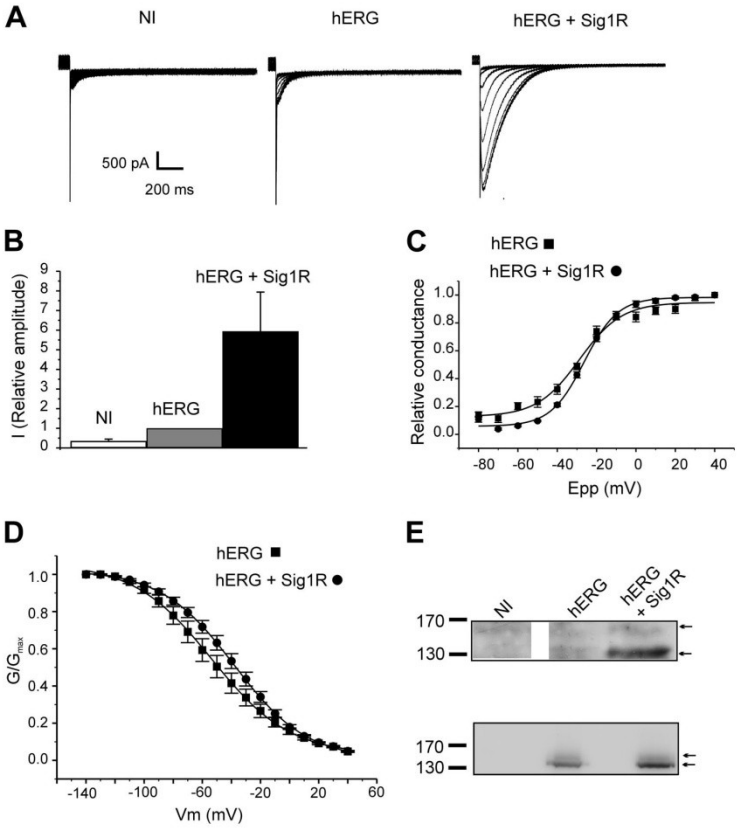

FIGURE 3. **Sig1R expression stimulates hERG currents in *Xenopus* oocytes.** *A*, families of tail currents recorded in noninjected (*NI*), *hERG* cRNA-injected (*hERG*; 25 pg/oocyte), and *hERG* + *Sig1R* cRNA-injected (hERG + Sig1R; 25 pg and 5 ng/oocyte, respectively) oocytes (representative experiment). The voltage protocol is given in the description of Fig. 1*C*. *B*, relative current amplitudes at −120 mV in noninjected, *hERG* cRNA-injected, and *hERG* + *Sig1R* cRNA-injected oocytes (arbitrary units, 1 corresponding to the mean current recorded in *hERG* cRNA injected oocytes). The values are the means ± S.E. of six independent experiments. *C*, voltage-dependent activation curves in hERG cRNA-injected (*black squares*) and *hERG* + *Sig1R*-cRNA-injected oocytes (*black circles*). The plots were fitted with a Boltzmann function. The values are the means ± S.E. of six independent experiments. *D*, voltage-dependent inactivation curves in *hERG* cRNA-injected (*black squares*) and *hERG* + *Sig1R* cRNA-injected oocytes (*black circles*). For the detailed voltage protocols, see "Materials and Methods." The values are the means ± S.E. of $n = 8$ independent experiments. *E*, *upper panel*, Western blots probed with a hERG antibody in noninjected (*NI*), *hERG* cRNA-injected (*hERG*; 25 pg/oocyte), and *hERG* + *Sig1R* cRNA-injected (hERG + Sig1R; 25 pg and 5 ng/oocyte, respectively) oocytes. *Lower panel*, Western blot of the same experiment using an identical concentration for *hERG* and *Sig1R* cRNA (15 ng/oocyte). Each Western blot is representative of three independent experiments.

TABLE 1

hERG voltage-dependent activation and inactivation parameters in *Xenopus* oocytes in the absence or presence of Sig1R

Steady-state activation and inactivation were fitted with the Clampfit software using the following Boltzmann function: $G/G_{max} = 1/(1 + e((V_{½} - V)/k))$. NS, not significant.

	Activation		Inactivation	
	$V_{½}$	Slope (k)	$V_{½}$	Slope (k)
hERG ($n = 6$)	−28.7 ± 0.3 mV	10.2 ± 2.7	−67.8 ± 9.1 mV	−22.2 ± 0.1
hERG + Sig1R ($n = 8$)	−26.8 ± 1.0 mV	9.2 ± 0.44	−64.7 ± 6.6 mV	−17.9 ± 0.8
Mann-Whitney	NS	NS	NS	NS

tration as those used for voltage-clamp experiments shown in Fig. 3*A*. *hERG* cRNA injection gave rise to a merely detectable protein expression (Fig. 3*E*). By contrast, when *hERG* and *Sig1R* cRNAs were co-injected, both mature (155 kDa) and immature (135 kDa) hERG glycoforms were clearly resolved (Fig. 3*E*) (Ficker *et al.*, 2003), correlating the current densities detected in the same conditions (Fig. 3*A*). The same potentiating effect was observed when *hERG* and *Sig1R* cRNA were injected at the same concentrations (Fig. 3*E*, *lower panel*).

Sig1R Silencing Modulates hERG Expression and Cell Adhesion to FN in K562 Cells—We further explored the function of Sig1R in K562 cells using a shRNA silencing strategy. K562 cells

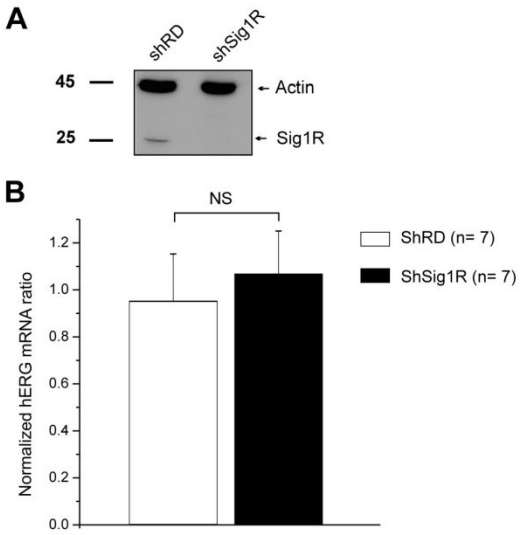

FIGURE 4. **Sig1R silencing does not alter *hERG* transcription in K562 cells.** *A*, Western blots probed with an anti-Sig1R or actin antibodies in shRD and shSig1R cells. *B*, *hERG* mRNA quantitative expression in shRD and shSig1R K562 cells. *hERG* mRNA levels were normalized to 36B4 ribosomal protein mRNA. *NS*, not significant (Student's *t* test).

were transduced with lentivirus containing either a random shRNA or a *Sig1R*-directed shRNA, yielding two cell populations named, respectively, shRD and shSig1R. Western blot experiments revealed a dramatic decrease in Sig1R expression in the shSig1R cell line (Fig. 4*A*). Quantitative PCR experiments revealed that Sig1R repression had no effect on *hERG* mRNA contents (Fig. 4*B*), ruling out any regulation of *hERG* transcription by Sig1R expression. Patch clamp experiments showed that the mean tail current amplitude recorded in shSig1R cells was clearly reduced when compared with shRD cells (Fig. 5*A*). At +40 mV, current density was 29.5 ± 1.7 pA/pF in shRD cells ($n = 40$) and 18.1 ± 1.4 pA/pF in shSig1R cells ($n = 45$, $p < 0.0001$, Mann-Whitney). Interestingly, Sig1R extinction neither significantly shifted voltage dependence activation (Fig. 5*B* and Table 2), nor modified deactivation kinetic time constants (Fig. 5*C*). The resting potential was not modified by Sig1R silencing (−24.5 ± 3.1 mV ($n = 13$) and −25.5 ± 3.8 mV ($n = 12$) for shRD and shSig1R K562, respectively, NS, Mann-Whitney), suggesting that hERG does not participate to the resting potential through a tonic window K^+ current.

We then explored the plasma membrane expression of the channel by performing extracellular labeling of hERG in non-permeabilized K562 cells. Flow cytometry analysis demonstrated that hERG membrane expression was significantly reduced in Sig1R-silenced cells (35.5 ± 0.1%; Fig. 5*D*), demonstrating that Sig1R regulates hERG current density through the number of ion channel at the plasma membrane. As expected, the decrease of hERG current density and membrane expression was accompanied by an inhibition of K562 cell adhesion to FN in shSig1R compared with the shRD population (≈40%; Fig. 5*E*).

At the protein level, Western blots using a pan-hERG antibody revealed that K562 cells express two different isoforms of hERG, *i.e.* hERG1a, corresponding to the full-length protein, and a N-terminal-truncated splicing variant hERG1b (29–31). We mainly detected the mature hERG1a isoform, a 155-kDa band representing the fully glycosylated form (mature hERG 1a), whereas the 135-kDa core-glycosylated immature form of hERG1a was seldom detected (Fig. 6*A*, *left panel*). By contrast, the hERG1b spliced isoform constantly appeared as two distinct bands, the 95-kDa fully glycosylated mature and the 80-kDa core-glycosylated immature forms (Fig. 6*A*, *left panel*). Both hERG mature glycoforms are known to represent the fraction of core-glycosylated channel subunit that has exited the ER to further process through the Golgi and reach the plasma membrane (32, 33). Sig1R silencing strikingly altered the hERG expression pattern. We observed in shSig1R cells a significant decrease in both hERG1a and 1b mature forms. In the same time, SigR silencing induced a dramatic increase in the hERG1b immature form (Figs. 6*A*, *left panel*, and 7*B*). Further analysis indicated that Sig1R silencing significantly reduced the apparent hERG1b maturation, which was quantified as the ratio $hERG1b_{mature}/hERG1b_{total}$ (33) (Fig. 6*C*), without significantly modifying the total amount of hERG (Fig. 6*D*).

The apparent effect of Sig1R on channel maturation was further explored in HEK cells transduced with either *hERG* + *cmycGFP* (control experiments) or *hERG* + *cmycSig1R*. In a first set of experiments, we successfully co-immunoprecipitated cmycSig1R with hERG, demonstrating a molecular interaction between the two proteins. Interestingly, Sig1R was associated with both mature and immature forms (Fig. 7*A*). We next studied hERG time course maturation processing by performing pulse-chase experiments. This revealed that maturation of hERG was increased in Sig1R-overexpressing cells (Fig. 7*B*). Quantitation revealed that newly synthesized immature hERG disappeared faster in Sig1R cells than in control cells (Fig. 7*C*), thus correlating with the appearance of the mature forms (Fig. 7*D*). We then analyzed the stability of the mature hERG in both Sig1R expressing and control cells. This revealed that mature hERG, most likely localized at the plasma membrane, was more stable in Sig1R expressing cells than in control cells (Fig. 7*E*). This apparent stabilization therefore correlated with enhanced biogenesis and current density potentiation in K562 and *Xenopus* oocytes.

Finally, we addressed the question of the mechanism involved in the sigma ligand-induced inhibition of hERG current. Igmesine (30 min of incubation, 10 μM) significantly reduced hERG current density in shRD K562 cells but produced no effects in shSig1R cells (Fig. 8*A*), demonstrating a specific effect through Sig1R. However, the same igmesine incubation protocol did not inhibit hERG membrane expression measured by flow cytometry in K562 (Fig. 8*B*), suggesting that the current inhibition produced by sigma ligands is not the result of an altered membrane channel stability. Using HEK 293 cells stably expressing both hERG and Sig1R, co-IP experiments revealed that igmesine incubation did not reduce the association between hERG and Sig1R (Fig. 8*C*).

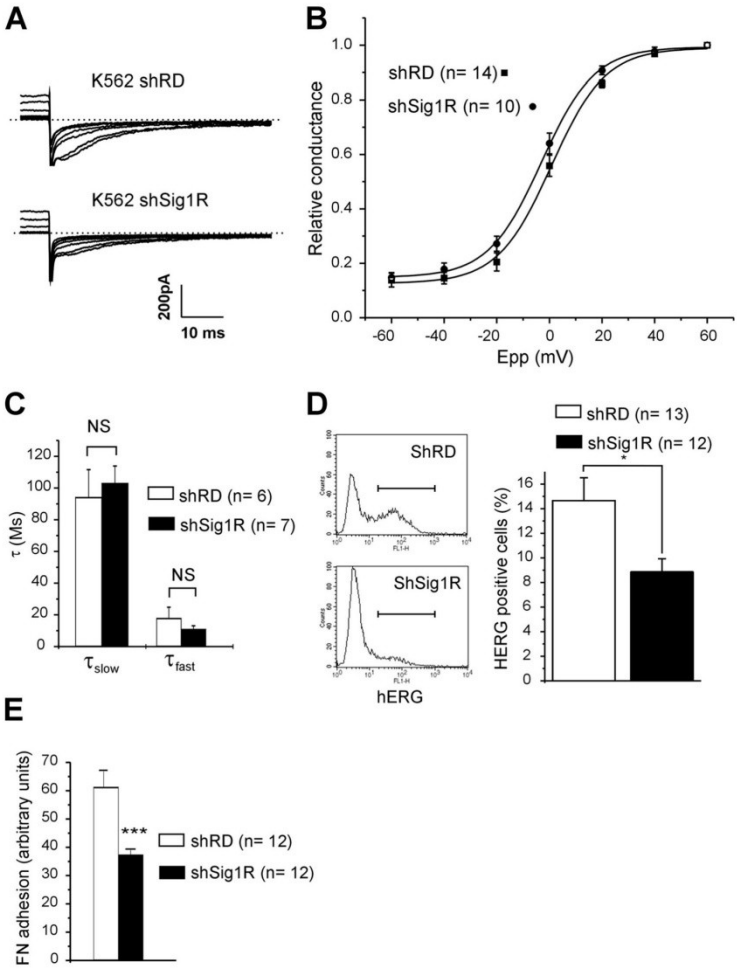

FIGURE 5. **Sig1R silencing reduces hERG current density in K562 cells.** *A*, families of hERG currents recorded in response to the voltage protocol described in the description of Fig. 1C. These representative traces were obtained from shRD K562 (K562 shRD, *upper panel*) and shSig1R K562 cells (K562 shSig1R, *lower panel*). *B*, mean activation plots obtained from shRD K562 (*black squares*) and shSig1R K562 cells (*black circles*). The plots were fitted using a Boltzmann function. *C*, deactivation rate constant of hERG current at −120 mV in shRD K562 (*white bars*) and shSig1R K562 cells (*black bars*). Deactivation was fitted with a double-exponential function. The values are the means ± S.E., Student's *t* test. *D*, *left panel*, surface expression of hERG by flow cytometry in shRD and shSig1R K562 cells (representative experiment). *Right panel*, corresponding histogram. *, $p < 0.02$, Mann-Whitney. *E*, FN adhesion in shRD (*white bar*) and shSig1R (*black bar*) K562 cells. The values are the means ± S.E. of 12 independent experiments. ***, $p < 0{,}001$ (Student's *t* test). In a single experiment, each value is the mean of three distinct wells.

DISCUSSION

The function of Sig1R is poorly understood, and its main endogenous ligand remains unknown. However, the acute effects of sigma ligands on K^+, Ca^{2+}, Na^+, and Cl^- currents suggest a functional link between Sig1Rs and ion channels from different molecular families, a hypothesis supported by several studies showing that Sig1Rs physically bind ion channel subunits (1, 5, 34, 35). We have previously demonstrated that sigma ligands block leukemia and lung cancer cell cycle in the G_1 phase through the inhibition of Kv1.3 and volume-regulated Cl^- channels (7, 8). In the present study, we have explored the putative link between hERG and Sig1Rs. hERG is a K^+ channel involved in cardiac repolarization that is also abnormally expressed in several cancer cells including leukemia. Physically associated with integrins and VEGF receptors, hERG enhances tumor cell progression through the regulation of cell/ECM interaction (20, 21).

Although the ability of sigma ligands to inhibit ion currents is well known, the primary activity of Sig1Rs at the level of ion channels is not understood. To address this question, we per-

TABLE 2

hERG kinetic properties in shRD and shSig1R K562 cells

Steady-state activation was fitted with the Clampfit software using the following Boltzmann function: $G/G_{max} = 1/(1 + e((V_{½} - V)/k))$. Deactivation kinetics were fitted with the following double-exponential function: $f(t) = A_1 e^{-t/\tau_{slow}} + C_1 + A_2 e^{-t/\tau_{fast}} + C_2$. NS, not significant.

	$V_{1/2}$	Slope (k)	Deactivation
shRD K562	−0.5 ± 1.9 mV (n = 15)	9.7 ± 0.7 (n = 15)	τ_{slow} = 94.1 ± 17.5 ms (n = 6) τ_{fast} = 17.8 ± 7.0 ms (n = 6)
shSig1R K562	−2.1 ± 1.6 mV (n = 10)	9.7 ± 1.6 (n = 10)	τ_{slow} = 103.0 ± 29.1 ms (n = 7) τ_{fast} = 10.9 ± 2.2 ms (n = 7)
Mann-Whitney	NS	NS	NS

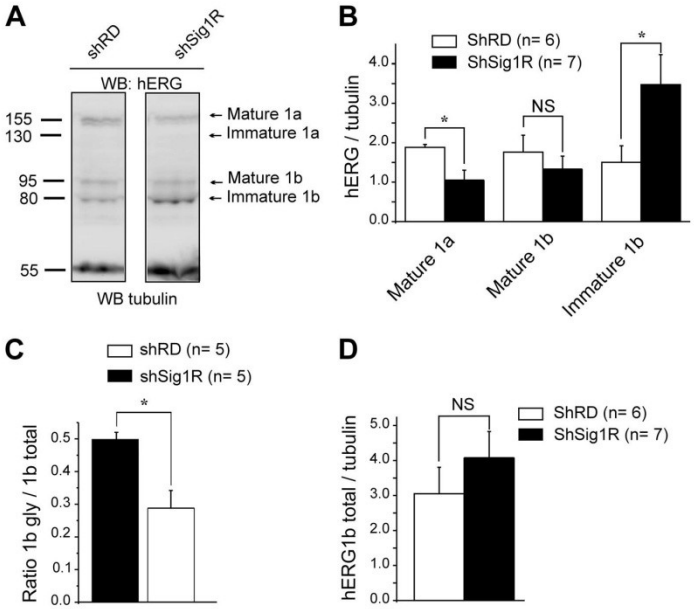

FIGURE 6. **Sig1R silencing alters hERG protein expression in K562 and cells.** *A*, Western blots (*WB*) probed with the anti-pan hERG or anti-tubulin antibodies, performed in shRD and shSig1R K562 cells. *B*, histogram of the densitometric analysis of mature and immature hERG isoforms in shRD (*white bars*) and shSig1R K562 cells (*black bars*). The values correspond to the densitometric ratio hERG/tubulin. *C*, trafficking efficiency of hERG1b in shRD and shSig1R (*black bar*) in K562 cells. Trafficking efficiency is calculated as the densitometric ratio (mature)/(mature + immature). *D*, histogram showing the total amount of hERG1b in shRD and Sig1R K562 cells. *, $p < 0,05$ (Mann-Whitney). *NS*, not significant.

formed heterologous co-expression of hERG and Sig1Rs in *Xenopus* oocytes and observed that Sig1Rs strongly increased current density without altering voltage-dependent activation and inactivation parameters. Accordingly, silencing Sig1Rs in K562 cells resulted in a reduction in current density, whereas other parameters (*i.e.* open probability and deactivation rate) remained unchanged. Hence, these results demonstrate for the first time that Sig1R expression stimulates ion currents. Our results, however, contrast with a previous report showing that Sig1R accelerated the inactivation rate of Kv1.4 in *Xenopus* oocyte (5), indicating that interacting modalities may depend on the channel type.

hERG current is mainly regulated by modifications of voltage-dependent and kinetic parameters but also results from the biogenesis/degradation ratio of subunits forming functional membrane channels (32, 36). We show herein that Sig1R expression enhanced hERG protein level in *Xenopus* oocytes, suggesting that Sig1R stimulates the current density by increasing the number of channels at the plasma membrane. This hypothesis was confirmed by the silencing of Sig1Rs in K562, inducing a decrease in hERG membrane labeling measured by flow cytometry. Accordingly, the level of the mature isoforms of hERG, *i.e.* hERG1a (full-length isoform) and hERG1b (N-terminally truncated isoform), which co-assemble in heterotetramers to form functional channels (33), was reduced in shK562 cells. Interestingly, Sig1R silencing did not alter *hERG* mRNA production in K562 cells, but Sig1R co-immunoprecipitated with hERG in HEK cells transduced with both proteins. In a whole, these data reveal that Sig1Rs control the post-translational biogenesis of hERG. Our attention turned therefore on the mechanism involved in the Sig1R-induced current potentiation. The analysis of hERG expression patterns in K562 cells silenced for Sig1R revealed a reduced maturation of both hERG1a and hERG1b isoforms, very similar to the profile described in hERG-expressing CHO cells and treated with Hsp90 inhibitors (32). This analogy suggests that

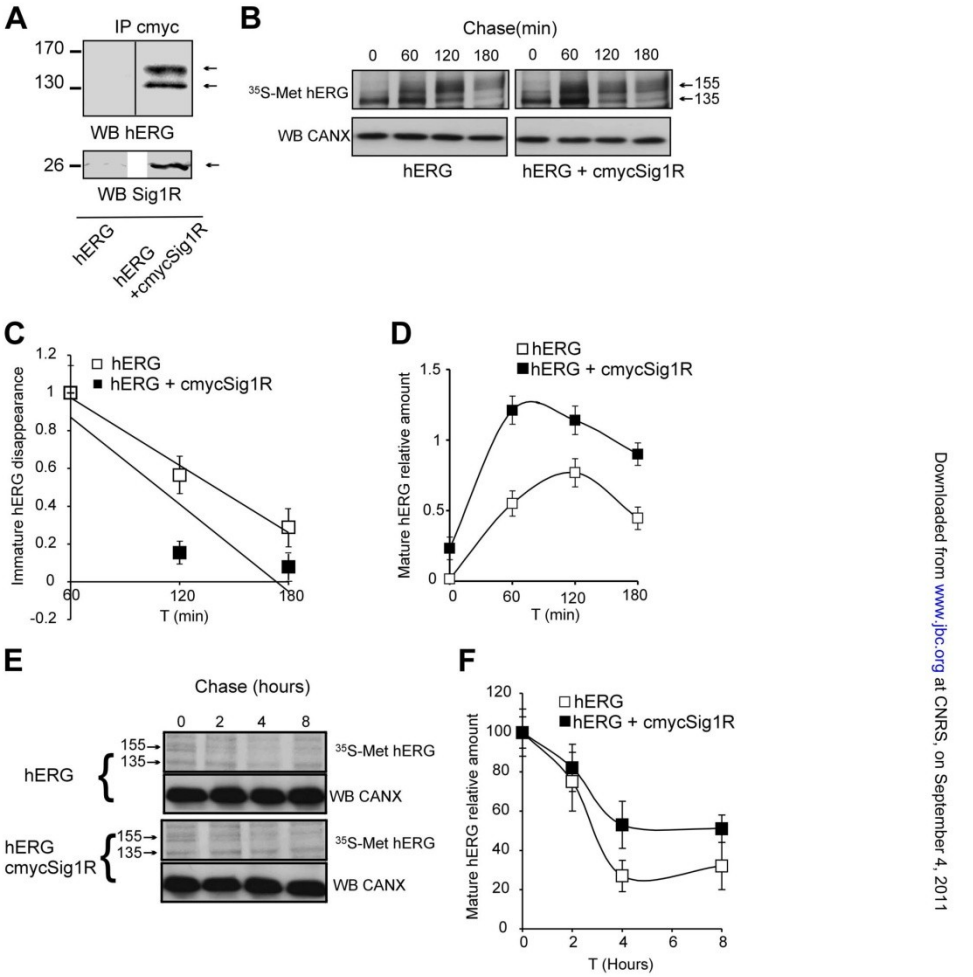

FIGURE 7. **Sig1Rs enhance hERG subunit maturation and stability in HEK cells through a direct interaction.** *A*, immunoprecipitation (*IP*) of HEK 293 cell lysate proteins with an anti-c-Myc antibody performed in cells transduced with *hERG* + *cmyc-GFP* as control (HEK + hERG) or *hERG* + *cmycSig1R* (hERG + cmycSig1R) and probed with anti-hERG (*upper panel*) or anti-Sig1R (*lower panel*) antibodies. *B*, 10-min [^{35}S]methionine pulse followed by up to 3-h chase experiments to analyze hERG maturation in the same cells as in *A*. Radiolabeled hERG was immunoprecipitated and visualized by fluorography following separation by SDS-PAGE. *C*, quantitation of immature hERG disappearance based on the experiments presented in *B*. The disappearance rate was determined between 60 and 180 min of chase. Quantitation was performed on nine experimental points obtained in two independent experiments and represented as the means ± S.E. *D*, quantitation of mature hERG appearance based on the experiments presented in *B*. Quantitation was performed on nine experimental points obtained in two independent experiments and represented as the means ± S.D. *E*, 1-h [^{35}S]methionine pulse followed by up to 8-h chase experiments to analyze hERG stability. Radiolabeled hERG was immunoprecipitated and visualized by fluorography following separation by SDS-PAGE. *F*, quantitation was performed on nine experimental points obtained in two independent experiments and represented as the means ± S.E. based on the experiments presented in *E*.

Sig1Rs potentiate ER/Golgi translocation of channel subunits, leading to an increase in the number of functional channels at the plasma membrane. The function of Sig1R in hERG maturation is consistent with its association with the ER-resident 135-kDa channel immature form and was further confirmed by pulse-chase experiments in HEK cells showing that Sig1R expression accelerated hERG rate of maturation. Sig1R can then be proposed as a candidate for the group of proteins controlling hERG trafficking such as Hsp/c70, Hsp90, or the KCNE1 K^+ channel β subunit (32, 37). Consistent with a function in hERG folding and/or maturation, in NG108-15 cells, Sig1Rs are associated at the mitochondria-associated ER membrane to the ER chaperone BiP, belonging to the Hsp70 family (11).

Furthermore, we found that Sig1R also associates with the 155-kDa mature form and potentiates its stability. Because the

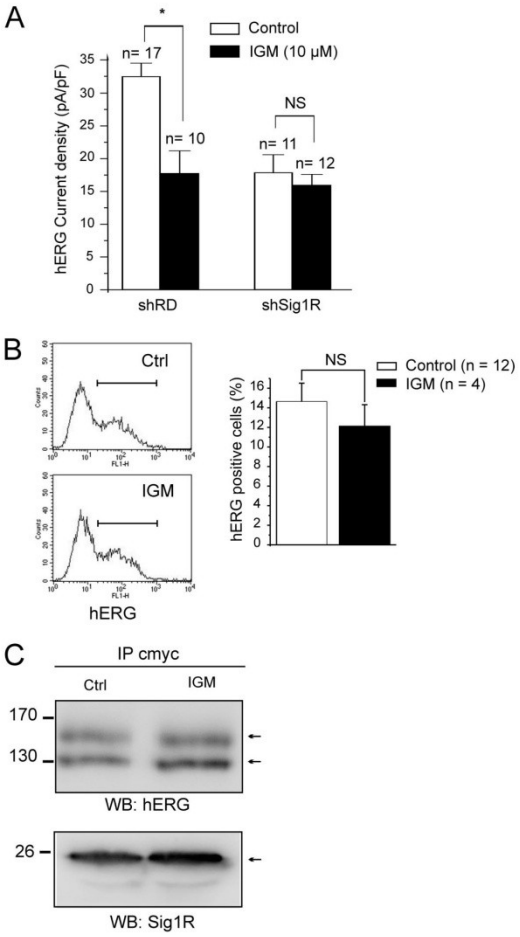

FIGURE 8. **Sigma ligands inhibit hERG current without decreasing hERG membrane expression or altering Sig1R-hERG association.** *A*, effect of cell incubation with igmesine (*IGM*, 10 μM, 30 min) on hERG current density in shRD and shSig1R K562 cells. *, $p < 0.002$, Mann-Whitney. *B*, *left panel*, effect of with igmesine (*IGM*, 10 μM, 30 min) on hERG surface expression by flow cytometry in shRD K562 cells. *Right panel*, corresponding histogram. *NS*, not significant, Mann-Whitney. *C*, effect of HEK 293 cell incubation with igmesine (*IGM*, 10 μM, 30 min) on Sig1R-hERG association. Immunoprecipitation (*IP*) with an anti-c-Myc antibody of HEK 293 cell lysate proteins. Western blots (*WB*) were probed with anti-hERG (*upper panel*) or anti-Sig1R (*lower panel*) antibodies. The cells were transduced with *hERG* + *cmycSig1R*. Representative of three independent experiments. *Ctrl*, control.

mature form represents the membrane functional channel (38), it can be proposed that Sig1R stimulates hERG current density by synergetically promoting hERG maturation and lowering channel membrane recycling.

Acute application of sigma ligands induced a reversible inhibition of hERG. The overall effect observed corresponds to a reduction in current density, as shown by the inhibition elicited by igmesine in conditions where the tail current represents the maximal activation state ($P_o = 1$) and inactivation is fully removed. This observation is consistent with previous reports showing that sigma ligands mainly inhibit current density associated with Kv1.3, voltage-dependent Na^+ channels, volume-regulated Cl^- channels, or acid-sensing ion channels (7, 8, 10, 12, 14, 15). It was thereby tempting to postulate that sigma ligands inhibit hERG currents by disrupting Sig1R from hERG subunits, leading to a decrease in the number of hERG surface expression. However, although igmesine decreased current density in K562 cells in a Sig1R-dependent manner, the ligand failed to significantly reduce hERG membrane labeling using the same sigma ligand incubation protocol. Moreover, the direct association between the two proteins expressed in HEK cells was not reduced by igmesine, as shown by co-immunoprecipitation experiments. It can then be concluded that sigma ligands do not inhibit hERG current by disrupting the Sig1R-channel complex and further plasma membrane expression. Sigma ligands are known to provoke a rapid redistribution of sigma receptors localization (39–41). Interestingly, cell treatment with the Sig1R agonist SKF 10,047 provoked the exclusion of Sig1Rs and associated proteins from lipid rafts, likely through a competition with cholesterol at Sig1R sterol-binding sites (42). Sigma ligands may thus induce the redistribution of the Sig1R-hERG complex within the plasma membrane leading to alteration in channel function without modifying plasma membrane expression (43).

Although the exact mechanism by which sigma ligands regulate hERG current will require further investigation, to our knowledge our data unravel the first mechanical and physiological link between Sig1R and ion channel function. The role of Sig1R on channel maturation and trafficking leads to reconsideration of the intrinsic function of sigma receptors in brain or heart but also in cancer. In many tumors, abnormally expressed hERG subunits associate with β_1 integrin and VEGF receptors to form channel signaling macrocomplexes involved in cell proliferation, invasiveness, and chemotherapy resistance (20, 29, 44). Understanding the regulation of hERG in cancer cells thus represents a question of paramount importance. In the human leukemic preosteoclastic cell line FLG 29.3, cell binding to FN transiently hyperpolarized membrane potential through hERG activation to potentiate integrin signaling machinery (45, 46). These results indicate that hERG activation is a necessary signaling step in the adhesion process. We show herein that the reduction of hERG current density by either Sig1R silencing or sigma ligands was accompanied by a reduction of the specific K562-cell FN adhesion. Moreover, igmesine and E-4031 produced no additive effects, demonstrating that Sig1Rs modulate cell/ECM interaction through the regulation of hERG channels. In K562 cells expressing both $\alpha v\beta 3$ or the native $\alpha 5\beta 1$, depolarization has been shown to enhance FN adhesion (47). Thus, it could be argued that hERG inhibition by either sigma ligands or Sig1R silencing would depolarize cells, potentiating FN adhesion. Nevertheless, E-4031 (47) and Sig1R silencing (our study) did not modify K562 resting potential, indicating that hERG inhibition cannot lead to FN adhesion through depolarization. It can then be proposed that Sig1R inhibition (by either gene silencing or pharmacological inhibition by ligands) or E-4031 leads to a reduced hERG participation to the $\beta 1$-integrin signaling complex during the FN adhesion process

(45). Interestingly, it has been demonstrated that members of the ether-à-gogo channel family could potentiate cancer cell invasiveness in a K^+ flux-independent manner, suggesting a mechanism independent of the regulation of membrane resting potential (48).

Altogether, our results unravel the regulating function of Sig1Rs on hERG expression. Sig1R may thus be considered as a new pharmacological target to reduce the activity membrane signaling channel macrocomplexes involved in cancer progression.

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3. Discussion :

Au cours de cette étude, j'ai mis en évidence une relation entre Sig1R et le canal potassique voltage-dépendant hERG dans un modèle de lignée cellulaire issue de leucémie myéloïde chronique, K562. Dans ce modèle, l'expression de Sig1R favorise l'expression à la surface du canal hERG en agissant sur la maturation et la stabilité de la forme mature du canal sans pour autant affecter l'expression globale de hERG (Crottes et al., 2011).

Cette étude est la première à mettre en évidence le rôle de Sig1R sur le trafic intracellulaire et l'adressage d'un canal ionique à la membrane plasmique, qui plus est dans un modèle cancéreux. Récemment, Kourrich et al. ont observé que l'activation de Sig1R par la cocaïne favorise l'expression à la surface des neurones du canal Kv1.2 (Kourrich et al., 2013), confirmant ainsi nos observations.

Sig1R régule un grand nombre de canaux ioniques différents de façon assez hétérogène (III.E). Cela suggère que Sig1R peut avoir plusieurs mécanismes d'action sur les canaux ioniques. Dans notre étude, nous observons ainsi que Sig1R favorise la densité de courant généré par le canal hERG en agissant à la fois sur sa maturation et son adressage à la membrane plasmique mais aussi sur la stabilité de cette forme mature, probablement en inhibant le recyclage du canal ionique. De la sorte, nos résultats soutiennent l'idée que Sig1R a plusieurs mécanismes d'action sur les canaux ioniques.

Néanmoins, de nouvelles questions se posent : comment Sig1R peut-il avoir des mécanismes d'action différents sur la même protéine ? Comment Sig1R peut-il réguler le trafic intracellulaire d'un canal ionique et dans le même temps réguler sa stabilité ?

Dans notre étude, Sig1R s'associe à hERG sous ses différents états de glycosylation (forme « core-glycosylated » (immature) et « full glycosylation » (mature)) et n'intervient pas sur son expression. Ainsi, Sig1R a une interaction stable et prolongée avec le canal hERG tout au long de sa synthèse et régule ce canal de façon totalement post-transcriptionnelle. On peut imaginer que la protéine Sig1R, associée à hERG, agit à la manière d'un adaptateur universel. Sig1R mettrait en place une plateforme protéique permettant de favoriser l'interaction de hERG avec les différentes chaperonnes et vésicules de transport nécessaires à son trafic intracellulaire. Ces interactions facilitées par Sig1R augmenteraient l'efficacité de son adressage à la membrane plasmique. Suivant le même mécanisme, le complexe protéique créé par Sig1R et hERG pourrait, à l'inverse, réduire l'interaction de hERG avec les protéines de la machinerie de recyclage et favoriserait par le même biais sa stabilité à la membrane plasmique.

Cette hypothèse expliquerait nos résultats mais aussi l'hétérogénéité de l'action de Sig1R sur les canaux ioniques. En effet, selon les protéines associées au complexe Sig1R / canal ionique, Sig1R pourrait moduler les propriétés électrophysiologiques, le trafic intracellulaire ou la stabilité du canal ionique.

Toutefois, aucune étude n'a mis en évidence un lien entre le complexe Sig1R / canal ioniques et des protéines liées à la synthèse, au transport ou à la modulation de l'activité des canaux ioniques.

Dans notre étude, nous observons que l'expression de Sig1R module l'activité du canal ionique hERG dans une cellule cancéreuse. Ces résultats confirment les résultats précédents du laboratoire et indiquent que l'expression de Sig1R module l'activité des canaux ioniques dans les cellules cancéreuses (Renaudo 2007). Cela suggère que la fonction de Sig1R dans les cellules cancéreuses (voir II.I) peut être liée à son rôle sur les canaux ioniques. Dans ce sens, notre étude montre que l'expression de Sig1R module l'adhésion *in vitro* des cellules leucémiques sur la fibronectine de façon dépendante de l'activité du canal hERG.

Ces résultats sont cependant trop préliminaires pour conclure quand au potentiel pro-tumoral de Sig1R et le lien éventuel entre sa fonction régulatrice des canaux ioniques et le potentiel invasif des cellules cancéreuses.

B. Sig1R favorise l'invasivité des cellules cancéreuses en modulant l'activité électrique de la membrane plasmique induite par la matrice extracellulaire.

1. Introduction :

hERG participe au dialogue de la cellule tumorale avec son microenvironnement (V.A). Plus précisément, l'activité de hERG est liée à celle de la sous-unité β1 des intégrines, elle-même dépendante de l'interaction de la cellule tumorale avec la matrice extracellulaire (MEC) (Crociani et al., 2013b; Pillozzi et al., 2007; Pillozzi et al., 2011b). La matrice extracellulaire (MEC) est essentielle au développement tumoral et permet aux cellules tumorales d'acquérir un profil invasif et une résistance aux agents chimiothérapeutiques (Lu et al., 2012b; Wolf and Friedl, 2011).

Dans la première partie de mon travail, nous avons montré comment Sig1R module l'activité de hERG en stimulant la maturation et l'adressage membranaire des sous-unités α du canal.

L'identification de ce mécanisme suggère que Sig1R intervient potentiellement dans le dialogue qu'entretient la cellule leucémique avec la MEC à travers son action sur le canal hERG. Ainsi, l'expression de Sig1R pourrait favoriser le potentiel invasif des cellules leucémiques.

Dans cette seconde étude, nous avons voulu vérifier cette hypothèse en détaillant le rôle de l'expression de Sig1R sur l'interaction de la cellule leucémique avec la MEC. Nous nous sommes intéressés aux conséquences de ce dialogue (migration, invasion, angiogenèse tumorale, extravasation) mais aussi aux caractéristiques moléculaires de ce dialogue telles qu'elles ont été décrites (activité du canal hERG, interaction de hERG et de la sous-unité β1 des intégrines, activation de la voie de signalisation PI3K/Akt) par l'équipe du Dr. Annarosa Arcangeli (Cherubini et al., 2005; Pillozzi et al., 2007).

Pour répondre à ces questions, j'ai étudié le rôle de l'expression de Sig1R sur l'interaction des cellules K562 avec la MEC en observant ses effets sur hERG, sur la sous-unité β1 des intégrines, sur la voie de signalisation PI3K/Akt ainsi que sur la sécrétion du VEGF. En utilisant des approches *in vitro* et *in vivo*, j'ai identifié le rôle de Sig1R sur les fonctions de migration d'invasion, d'induction de l'angiogenèse et de dissémination des cellules leucémiques. Je me suis également intéressé à la validité de ces résultats dans le

cadre des cancers colorectaux pour lesquels l'interaction hERG/ β1 a été récemment décrite (Crociani et al., 2013b).

2. Article: Sig1R potentiates chronic myeloid leukemia cell invasiveness potency by shaping membrane cell electrical activity in response to ECM stimulation

Sig1R potentiates chronic myeloid leukemia cell invasiveness potency by shaping membrane cell electrical activity in response to ECM stimulation.

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Abstract

The sigma 1 receptor (Sig1R) is a stress-activated chaperone involved in stroke, neurodegenerative diseases and addiction. Sig1R shapes neuronal activity by regulating GPCR and ion channels in response to brain injury. Also detected in cancer cell, its functions in carcinogenesis remain elusive. Here we show that Sig1R is overexpressed in myeloid leukemias (ML) and colorectal cancers (CRC) and regulates cancer cell electrical signature by dynamically controlling membrane expression of hERG K^+ channels. Indeed, Sig1R promotes formation of hERG/β1-integrin signalling complexes upon extracellular matrix (ECM) stimulation, triggering of PI3K/AKT pathway. Consequently, in the presence of Sig1R in cancer cells, motility and VEGF secretion are increased. *In vivo*, Sig1R expression increases aggressiveness by potentiating invasion and angiogenesis. This study provides insights into a novel function of Sig1R as an actor of the crosstalk between cancer cell and their microenvironment, driving oncogenesis by shaping cell electrical activity in response to ECM.

Introduction

The sigma 1 receptor (Sig1R) is a 25 kDa chaperone protein mainly expressed in the brain and residing at the endoplasmic reticulum (ER)-mitochondria interface (mitochondria ER-associated membrane, MAM). Tissue injury leading to ER stress provokes Sig1R translocation from MAM to plasma membrane (PM) where it directly interacts with client proteins such as ion channels and G protein-coupled receptors (GPCR) to shape neuron activity and enhance cell survival (Hayashi and Su, 2007; Kourrich et al., 2013; Kourrich et al., 2012; Mavlyutov et al., 2013). In particular, Sig1R functionally interacts with ion channel from various families including voltage-dependent K^+, Na^+ and Ca^{2+} channels (Aydar et al., 2002; Balasuriya et al., 2012; Fontanilla et al., 2009; Soriani et al., 1998), volume-regulated chloride channels (VRCC), acid-sensing ion channels (ASIC) (Carnally et al., 2010) and NMDA receptors (Balasuriya et al., 2013; Maurice and Su, 2009). Therefore, Sig1R has been associated to many pathophysiological contexts including stroke, neurodegenerative diseases (amyotrophic lateral sclerosis, Alzheimer, retinal degeneration), pain or cocaine addiction (Kourrich et al., 2013). In addition, a growing number of evidence suggests that Sig1R is expressed in cancer cells (Crottes et al., 2013) but its function within the tumour tissue has not been elucidated.

During the past 10 years, the role of ion channels in cancer has arisen. Electrical signature of cancer cells is remodelled by the aberrant expression of channels often absent from the

healthy tissue, which in turn participate to hallmarks of cancer (Hanahan and Weinberg, 2011; Huang et al., 2012; Li and Hanahan, 2013; Prevarskaya et al., 2010). For example, hERG (human ether-à-go-go-related–gene, KCNH2), a voltage-dependent K^+ channel mainly involved in cardiac activity and CNS action potential firing (Vandenberg et al., 2012), has been characterized as a biomarker of many solid tumours (colorectal cancer, glioblastoma, head and neck cancers) (Crociani et al., 2014b; Masi et al., 2005) and acute or chronic leukemias (Pillozzi et al., 2007; Pillozzi et al., 2011a). By forming membrane protein platforms with receptors of the tumour cell microenvironment, such as integrins (adhesion receptors of the extracellular matrix (ECM)), hERG deeply influences signalling pathways controlling in turn cancer cell spreading (Becchetti et al., 2010). As the maturation and membrane expression of hERG is augmented by the presence of Sig1R through a direct physical interaction (Crottes et al., 2011), we wondered if Sig1R could mediate signalling events between hERG and the extracellular matrix in myeloid leukemia cells.

In this study, we have identified Sig1R as a key regulator in the ECM-induced formation of hERG-β1 integrin complex in K562 chronic myeloid cells, stimulating motility and VEGF secretion. At the molecular level, Sig1R increases hERG density at the plasma membrane, promoting the formation of hERG/Sig1R/β1 integrin complex and activating the AKT signalling pathway. *In vivo*, Sig1R silencing results in reduced tumour invasion, angiogenesis and extravasation. The mechanism was extended to colorectal cancer (CRC) in which Sig1R silencing decreased invasiveness potency in a hERG-dependent manner. Importantly, we show here that Sig1R, by remodelling cancer cell electrical signature, finely tunes cancer cell behaviour in response to input signals from the tumour microenvironment.

Results

FDM promotes hERG current density through a rapid Sig1R-dependent recruitment of channels at the surface

In leukemic cells, hERG current can be stimulated by α5β1 integrin receptors which are themselves activated by ECM components such as fibronectin (FN) (Pillozzi et al., 2007; Pillozzi et al., 2011a). Several ion channels including hERG are regulated by the Sig1R chaperone protein (Crottes et al., 2013; Crottes et al., 2011; Kourrich et al., 2013), but whether Sig1R participates to the dynamic regulation of ion channels in response to ECM stimulation is unknown. To address this question, patch-clamp experiments were performed to compare hERG current in control (shRD) and Sig1R-silenced (shSig1R) K562 CML cells (Fig. 1a) (Crottes et al., 2011). Currents were recorded 15 minutes to 3h after cell seeding

either on plastic dishes or on dishes coated with FN-rich fibroblast-derived 3D matrix (FDM), which mimicks *in vivo* mesenchymal matrices (Beacham et al., 2007; Cukierman et al., 2001; Goetz et al., 2011) with a composition similar to those observed in the bone marrow(Nair et al., 2010). In control cells plated on uncoated dishes, tail current density, recorded after a 60-mV preconditioning pulse to fully activate hERG (Crottes et al., 2011), peaked at 13.52 ± 2.02 pA/pF. Seeding cells on FDM increased hERG current density by around 96% (Fig. 1b, c). The increased current was observed 15 minutes after seeding and was maintained for at least 3h (Supplementary Fig. 1). Using the same protocol, Sig1R-silenced cells did not significantly change current density in uncoated conditions but FDM-induced stimulation was completely abolished (Fig. 1b, c). As expected, FDM-induced current stimulation was abrogated in the presence of a blocking antibody directed against the β1 integrin subunit (Fig. 1b, c). Note that the anti- β1 integrin subunit antibody had no effect on hERG current density in Sig1R-silenced cells. We next wondered whether FDM-stimulation of hERG current was accompanied by an increase in the number of ion channels at the plasma membrane and whether the phenomenon was Sig1R-dependent. Therefore, we performed flow cytometry to detect channels expressed at the plasma membrane using an antibody directed against an extracellular loop of the channel. We observed two populations of cells that we identified as "low" and "high" for hERG membrane expression levels. Similarly in patch-clamp experiments, we found that cell stimulation with FDM significantly shifted the distribution toward the "high" level population in control cells, increasing the percentage of “high” level population from 43.07 ± 4.62 to 63.52 ± 4.53 in presence of FDM (Fig. 1d, e). By contrast, FDM had no effect when Sig1R expression was repressed (40.66 ± 4.25 % and 46.51 ± 4.09 % in absence and presence of FDM, respectively; Fig. 1d, e). Considering that Sig1Rs directly bind ion channels to potentiate their trafficking to the plasma membrane(Balasuriya et al., 2012; Crottes et al., 2011; Kourrich et al., 2013), we wanted to verify whether FDM-induced stimulation also increased Sig1R surface expression. As the N-terminus of the Sig1R is extracellular, we transfected K562 cells with an N-terminal GFP-fused Sig1R to measure the Sig1R surface expression by flow cytometry. As expected, FDM significantly augmented the proportion of cells expressing Sig1R at the PM (12.33 ± 0.92 % and 17.03 ± 2.07 % in absence or the presence of FDM, respectively; Fig. 1f, g).

Previously, we showed that Sig1R increased steady state hERG current density by enhancing channel maturation ratio (Crottes et al., 2011). We wondered whether FDM could transiently stimulate hERG maturation and whether this effect could be abrogated by Sig1R suppression. Western blot experiments, performed on cells plated 30 min on either plastic or FDM, revealed that FDM-induced stimulation of the current was not due to a transient stimulation of hERG maturation (Supplementary Fig. 2a, b). Altogether, these results show

that FDM induces a rapid recruitment of hERG channels to the plasma membrane in a Sig1R-dependent manner.

Sig1R promotes the formation of FDM-induced β1-integrin / hERG complexes

It is known that hERG current stimulation by ECM in leukemic cells is accompanied by the binding of channels to β1 integrin subunits (Pillozzi et al., 2007; Pillozzi et al., 2011a). We therefore asked whether Sig1R was involved in the formation of such channel signalling macrocomplexes at the plasma membrane. In K562 cells transiently transfected with GFP-hERG and stained for extracellular β1 integrin, confocal microscopy experiments showed that FDM induced at 30 min a 3.5-fold increase in hERG co-localisation with β1 integrin subunit at the plasma membrane (Fig. 2a, upper panel). However, this effect was abolished completely by Sig1R silencing (Fig. 2a, middle panel). In a second set of experiments, we transiently transfected K562 cells with GFP-Sig1R and observed that FDM also provoked a 2.5-fold increase in β1 integrin / Sig1R co-localisation after 30 min (Fig. 2a, lower panel). In parallel to these experiments, we used three previously established HEK293T cell lines stably expressing cmyc-Sig1R alone, hERG1a with c-mycSig1R and hERG1a with a shRNA targeting Sig1R (Crottes et al., 2011). As the β1 integrin subunit is constitutively expressed in HEK293 cells, we performed immunoprecipitation of this protein followed by immunodetection of hERG and cmycSig1R. We observed that both hERG and cmycSig1R co-immunoprecipitated with β1 integrin; interestingly, in the absence of Sig1R, only low levels of hERG could be detected after β1 integrin immunoprecipitation (Supplementary Fig. 3). These data strongly suggest that Sig1R is necessary to promote the hERG/β1 integrin interaction and that Sig1R itself is included within the complex. To further confirm this mechanism, we performed a flow cytometry-based FRET assay to detect hERG/β1 integrin physical interaction at K562 cell surface (Banning et al., 2010). To assess a correct FRET signal, K562 cells stained with only one of both fluorochromes were used as control to determine the FRET-positive gate (FRET+). β1-integrin and hERG were respectively stained with AlexaFluor 488-conjugated and AlexaFluor 594-conjugated antibodies and were used as donor and acceptor, respectively (Fig. 2b). Compared to uncoated conditions, FDM induced after 30 min a significant increase in FRET-positive cells (17.7 ± 1.5% and 31.5 ± 5.3% in uncoated and FDM-coated conditions, respectively; $p < 0.05$). As expected, the effect of FDM on β1 integrin/hERG direct interaction was completely abolished in Sig1R-silenced K562 (Fig. 2c). Moreover, we observed that FDM or Sig1R silencing has no effect on β1 integrin surface expression (Supplementary Fig. 4). Altogether these data indicate that the

formation of integrins/hERG channel membrane complexes in response to ECM is driven by Sig1R in leukemic cells.

Sig1R silencing inhibits FDM-triggered AKT signalling pathway

The formation of hERG/β1 integrin platforms in response to ECM is known to trigger PI3K/AKT signalling pathways in acute leukemias and colon cancer cells (Pillozzi et al., 2007; Pillozzi et al., 2011a). Therefore, we wondered if Sig1R inhibition could alter AKT phosphorylation in the context of K562 cell interaction with FDM. Western blot experiments showed that in control K562 cells, the relative proportion of phosphorylated-AKT represents 40.48 ± 8.57 % of total AKT on uncoated dishes, a value that increased up to 86.56 ± 11.37 % for FDM-challenged cells (Fig. 3). As expected, inhibiting hERG current using E-4031 (a specific blocker of hERG; 50 μM) completely abolished FDM-dependent AKT activation (Fig. 3). In the same manner, Sig1R silencing suppressed FDM-induced AKT phosphorylation (Fig. 3). Interestingly, E-4031 had no effect on FDM-stimulated Sig1R-silenced cells, pointing out the absence of any additive effect between Sig1R and hERG inhibitions on AKT activation (Fig. 3). These results indicate that the pivotal role of Sig1R in β1 integrin/hERG channel complex formation at the plasma membrane is mirrored at the intracellular level by the activation of the AKT signalling pathway.

FDM stimulates cell migration and actin organization in a Sig1R manner.

In many cancers, up-regulation of AKT signalling is related to highly migrating cell phenotype (Chin and Toker, 2009; Pillozzi et al., 2007; Pillozzi et al., 2011a; Rodon et al., 2013; Virtakoivu et al., 2012). Having found that Sig1R controls hERG/β1 integrin/AKT pathway in response to FDM stimulation, we hypothesized that Sig1R inhibition could alter K562 cell migration potency. Thus, we performed time-lapse recording of K562 sh RD and sh Sig1R cells plated on either uncoated or FDM-coated dishes for 3h (Fig. 4 a, b). Cell motility was evaluated by measuring the distance travelled and the persistence of migration. We observed that FDM promoted cell motility by increasing the distance cell travelled from 20.7 ± 0.8 μm in uncoated conditions to 33.9 ± 2.1 μm in FDM-coated dishes (Fig. 4b). Persistence of migration was evaluated by measuring the Euclidian distance traveled during the lap of acquisition time. In control cells, FDM increased the distance traveled from 2.86 ± 0.16 μm to 10.62 ± 1.65 μm. The effects of FDM on both parameters were lowered by the hERG inhibitor E-4031 (50 μM) or the PI3 Kinase inhibitor LY294002 (10 μM; Fig. 4a, b), indicating the contribution of hERG and PI3 Kinase/AKT pathway in FDM-dependent cell migration

stimulation. Silencing Sig1R expression (sh Sig1R) significantly reduced FDM-induced motility; moreover, in sh Sig1R cells, E-4031 or LY294002 had weaker or non-significant effects on distance travelled or persistence of migration (Fig. 4a, b).

The AKT pathway has been linked to cell migration through the control of cell shape and actin cytoskeleton remodelling (Dillon and Muller, 2010; Friedl and Wolf, 2003). To address whether the presence of Sig1R in K562 cells would impact actin re-organisation following seeding on FDM, we imaged polymerized actin by confocal microscopy. After 3h, control cells seeded on uncoated dishes showed a low number of individual actin spikes at cell periphery (5.16 ± 2.43 spikes per cell; Fig. 4c). By contrast, FDM coating induced a 3-fold increase in spike number, an effect reversed by E-4031 (50 µM) or LY294002 (10 µM) treatment (Fig. 4c). In sh Sig1R K562 cells, FDM failed to significantly increase the appearance of spikes when compared to uncoated condition (Fig. 4c). In addition, neither E-4031 nor LY294002 had any effect on Sig1R-silenced cells (Fig. 4c). We next wondered whether the apparition of actin spikes was accompanied by a FDM-dependent alteration in cell morphology. K562 cells were seeded on either uncoated or FDM-coated dishes and circularity was quantified using a coefficient varying between 1 (fully round cells, FR) and 0 (not circular, NC). Cells were then sorted according to a circularity threshold of 0.5 (see material and methods and supplementary Fig. 5a for representative images showing the diversity of morphologies of K562 on FDM after 3h). We observed that FDM increased the proportion of NC cells as a function of time. This effect was completely abolished in sh Sig1R cells (Supplementary Fig. 5b). We next assayed the dependence of this parameter on hERG and AKT activities. In control cells, FDM increased the proportion of NC cells when compared to uncoated conditions (from 3.05 ± 3.05 % to 23.76 ± 3.95 %; Supplementary Fig. 5b, c). The effect of FDM on cell shape was diminished in the presence of either hERG (E4031, 50 µM) or AKT (LY294002, 10 µM) inhibitors (Supplementary Fig. 5c). In contrast, FDM only weakly increased the proportion NC cells in Sig1R-silenced K562 (from 0.34 ± 0.34 % in absence of FDM to 5.12 ± 1.59 % in presence of FDM; Supplementary Fig. 5c). In the latter case, E-4031 and LY294002 had no significant effects on sh Sig1R plated on FDM when compared to controls cells (Supplementary Fig. 5c). Taken together, these results suggest that Sig1R drives the FDM-induced morphological changes of K562 through its interaction with hERG and PI3K/AKT.

Sig1R promotes FDM-induced cell motility *in vitro* and cell invasion *in vivo*.

We next wanted to know the consequences of Sig1R down-regulation *in vitro.* To address them, we used a model of tumour xenotransplantation in zebrafish embryo (Fig. 5a) (Marques et al., 2009). As shown in Fig. 5b, the silencing of Sig1R expression reduced K562 cell invasion in zebrafish embryos by about 42 % when compared to control shRNA (Fig. 5b), confirming the role of Sig1R on cancer cell invasion *in vivo*.

hERG/β1 integrin macrocomplexes are also known to promote migration and invasion by stimulating VEGF secretion and further autocrine activation of Flt-1 receptor(Pillozzi et al., 2007). Moreover, in CML, VEGF secretion is regulated through the PI3K/AKT signaling pathway (Legros et al., 2004). We thus asked if Sig1R plays a role in tumour-induced angiogenesis. We observed that *in vitro,* Sig1R expression was strongly associated to VEGF secretion (Fig. 5c). The application of E-4031 inhibited VEGF secretion in control K562 cells and not in Sig1R-silenced cells, suggesting that Sig1R promoted VEGF secretion through the regulation of hERG channels activity. *In vivo*, using a zebrafish embryo-based model of tumour-induced angiogenesis (Nicoli and Presta, 2007) (Fig. 5d), we observed that the silencing of Sig1R reduced the ability of K562 to induce neoangiogenesis by about 50% (Fig. 5e).

To further explore the requirement of Sig1R for tumour cell invasiveness, we investigated the effect of Sig1R in the extravasation step of metastasis. We first observed *in vitro* that the silencing of Sig1R reduced the ability of K562 cells to transmigrate through an endothelial HUVEC monolayer by about 60%, as assayed in a Boyden chamber (Fig. 5f). Accordingly, using an *in vivo* model for tumour cell extravasation in NOD-SCID Hairless mice(Puissant et al., 2012) (Fig. 5g), we observed that Sig1R silencing decreased the number of K562 cell evading from the blood compartment into lung tissues by 80% when compared to control cells (Fig. 5h). Altogether, our results suggest a functional requirement of Sig1R in tumour cell invasion, neoangiogenesis and extravasation *in vitro* and *in vivo*.

Discussion

Our study unveils a new function for the Sig1R chaperone in oncogenesis. We demonstrate that Sig1R drives cancer cell behaviour by shaping membrane electrical properties in response to the extracellular matrix microenvironment. This effect is induced by a Sig1R-dependent mobilisation of voltage-dependent K^+ channels at the plasma membrane of leukemia cells in response to ECM stimulation. We show that Sig1R is required for the formation of a signalling complex at the plasma membrane of cells challenged with ECM, leading to AKT phosphorylation. The complex includes the β1 integrin subunit and hERG, a

K^+ channel characterized as a bad prognostic marker in several leukemias and solid tumours (Pillozzi and Arcangeli, 2010). Importantly, we show that the presence of Sig1R stimulates cell motility and VEGF secretion levels *in vitro* in a hERG- and AKT-dependent manner. Using zebrafish and mice xenograft models, we provide evidence that Sig1R increases invasiveness, angiogenesis and extravasation potency of chronic myeloid leukemia cells *in vivo*.

We find that CML cell stimulation by ECM induced a rapid increase in hERG current density. While this observation is consistent with previous studies showing that in AML, hERG current is stimulated by fibronectin, a major component of ECM (Cherubini et al., 2002), the mechanism involved was still unknown. Using flow cytometry and confocal microscopy, we reveal that this effect is likely the consequence of an increase in the number of channels at the plasma membrane. Importantly, we show that the fast recruitment of hERG following cell contact with ECM requires the presence of Sig1R. This suggests that Sig1R acts as a chaperone driving hERG to the plasma membrane in response to ECM stimulation. The concomitant increase in both hERG and Sig1R densities at the plasma membrane strengthens this idea. In agreement with this hypothesis, we and others have shown that Sig1R directly interacts with ion channels, promoting their activity and their plasmalemmal expression(Balasuriya et al., 2012; Kourrich et al., 2013). In particular, we have previously demonstrated that Sig1R promotes hERG surface expression by enhancing hERG steady-state maturatio n(Crottes et al., 2011). However, in the context of ECM stimulation presented here, hERG current was increased without modifying channel maturation ratio (Supplemental Fig. 2). This suggests that ECM triggers the trafficking of available mature hERG channels located at the vicinity of the plasma membrane, and that this effect depends on the presence of Sig1R. Accordingly, we previously observed that Sig1R not only co-immunoprecipitates with core-glycosylated ER-form hERG subunits, but also with the fully-glycosylated surface form of the channel (Crottes et al., 2011).

We report that ECM promotes a rapid colocalisation of the β1 integrin subunit with both hERG and Sig1R at the plasma membrane. What is more, Sig1R silencing reduces hERG/β1-integrin subunit physical interaction following cell contact with ECM as revealed by FRET assays. These results demonstrate that Sig1R is required for the dynamic formation of hERG/β1 integrin subunit complex in response to a physiological stimulation of cancer cells by ECM. Several reports have demonstrated that hERG/integrin interaction was required for the activation of signalling pathways triggered by cell contact with ECM. By promoting hERG/β1 integrin subunit interaction, it can be proposed that Sig1R is a key regulator of the dialogue between cancer cells and their microenvironment (Arcangeli and Yuan, 2011). This hypothesis is further confirmed at the cellular level by the fact that Sig1R silencing impaired

AKT phosphorylation occurring after cell adhesion to ECM, a transduction pathway triggered by the hERG/β1-integrin complex in acute leukemias and colorectal cancers (Arcangeli et al., 2012; Crociani et al., 2014b; Pillozzi et al., 2007).

We also provide evidence that Sig1R silencing decreased ECM-dependent cell motility not only *in vitro*, but also *in vivo* by using a zebrafish xenograft model (Feitsma and Cuppen, 2008; Yilmaz and Christofori, 2009). Interestingly, altered motility was paralleled by a reduced capacity for cells to reorganize their actin cytoskeleton and to change their shape following contact with ECM, two phenomena which are functionally linked to cell migration phenotype (Hall, 2009; Rao and Li, 2004; Yilmaz and Christofori, 2009). Together, these data reveal that Sig1R participates to CML cell migration potency in response to ECM. Whether this function is dependent on the formation of hERG signalling macrocomplex is confirmed by the fact that hERG and AKT pharmacological inhibitions mimicked Sig1R silencing in a non-additive manner. In a good agreement with this hypothesis, the formation of the hERG/β1 integrin subunit complex in response to FN increases cell migration ability in an AKT-dependent manner in leukemias (Pillozzi et al., 2007; Pillozzi et al., 2011a; Podar et al., 2002).

In the course of our study, we found that Sig1R silencing impaired VEGF secretion in CML cells. We hypothesize that this effect is the consequence of hERG regulation by Sig1R, as channel pharmacological blocking also reduced VEGF secretion. This idea is further confirmed by the following two observations: first, hERG/ β1 integrin subunit complex controls VEGF secretion in acute myeloid leukemia, colorectal cancer and glioblastoma (Crociani et al., 2014b; Masi et al., 2005; Pillozzi et al., 2007); Second, VEGF secretion is up regulated by AKT activation in CML cells (Legros et al., 2004), a pathway triggered by hERG in CML and CRC.

In a pathologic context, VEGF secretion sustains angiogenesis (Podar et al., 2002; Recher et al., 2004; Till et al., 2005) and cancer cell transendothelial migration (Chen et al., 2012; Jean et al., 2014; Six et al., 2002), thus increasing blood stream invasion and dissemination to distant organs. In this study, we demonstrate that the presence of Sig1R increases angiogenesis *in vivo*, but also stimulates transendothelial migration *in vitro* and lung extravasation *in vivo*. In other words, the presence of Sig1R in leukemia cells likely confers a proinvasive phenotype. We therefore interrogated the Oncomine database and found that Sig1R was significantly overexpressed in human CML and AML, but also in CRC when compared to corresponding healthy tissues (Supplementary Fig. 6a). Notably, hERG has been linked to enhanced invasion, angiogenesis and bad prognosis in these three cancer types (Crociani et al., 2014b; Crociani et al., 2013a; Lastraioli et al., 2004; Pillozzi et al.,

2007). Therefore, we explored the consequences of Sig1R silencing by sh RNA in HTC-116 cells. In this CRC cell line, hERG expression has been linked to angiogenesis and invasive properties (Crociani et al., 2013a; Lastraioli et al., 2004). We observed a hERG-dependent decrease in cell motility and VEGF secretion *in vitro* and a reduced invasive potency *in vivo* (Supplementary Fig. 6b-f). Altogether, these observations points out that our primary findings in CML can be extended to other cancers characterized by the expression of hERG as a biomarker. We thus hypothesise that Sig1R, by promoting hERG ion channel expression at the plasma membrane, increases invasive phenotype.

Recently, Sig1R has been implicated in the regulation of neuronal plasticity during neurologic disorders such as stroke, addiction or neurodegenerative diseases (Hayashi and Su, 2007; Kourrich et al., 2013; Kourrich et al., 2012; Mavlyutov et al., 2013; Prause et al., 2013; Ruscher et al., 2011). Indeed, the key cellular mechanisms linking Sig1Rs to neuronal survival involve the stress-induced translocation of Sig1Rs from the MAM to other parts of the cell, whereby Sig1Rs bind and modulate the activities of various ion channels and membrane receptors (Kourrich et al., 2012). Taken together, our results suggest that the basic protective function of Sig1R is hijacked to adapt tumour cell behaviour to the signals or the physical constraints occurring within cancerous tissues. In line with this new idea, we had previously shown that Sig1R modulates volume-regulated chloride channels in leukemia and small cell lung carcinoma cells to enhance resistance to apoptotic signals (Renaudo et al., 2007). Because the ion channel chaperoning activity of Sig1R can be regulated by exogenous compounds used as CNS drugs (sigma ligands) (Maurice and Su, 2009), the present study suggests that the Sig1R protein may be used as a therapeutic target to specifically alter ion channel activity in cancerous tissues.

Legends

Figure 1. FDM promotes a Sig1R-dependent recruitment of hERG at the plasma membrane. Control and Sig1R-silenced K562 cells (sh RD and sh Sig1R, respectively) were plated for 30 min on either uncoated or FDM-coated dishes. (**a**) Western blot showing Sig1R expression in sh RD and sh Sig1R K562 cells. (**b**) Representative hERG tail-current recorded from cells plated on uncoated or FDM-coated dishes in presence or absence of β1 integrin blocking antibody (1 µg / mL). hERG currents were measured at -120 mV during 5s after a 4s prepulse at +60 mV to fully activate hERG. (**c**). Corresponding current amplitude histogram. Values are mean ± S.E.M. (n = 7 to 19 cells; * $p < 0.05$; ** $p < 0.01$). (**d**) hERG channels expressed at the plasma membrane were labeled with an antibody directed against an extra-cellular loop of hERG channel; the percentage of hERG-positive cells was determined by flow cytometry. (**e**) Corresponding histogram showing hERG surface expression in each

condition. Values of the percentage of hERG-positive cells are mean ± SEM of 6 to 8 independent experiments (* $p < 0.05$; ** $p < 0.01$). (**f**) K562 transiently transfected with a GFP-Sig1R construct were labeled with an anti-GFP antibody without permeabilization and the percentage of extracellular GFP-Sig1R-positive cells was determined by flow cytometry. (**g**) Corresponding histogram showing GFP-Sig1R surface expression among GFP positive cells. Values are the mean ± SEM of 7 independent experiments (* $p < 0.05$).

Figure 2. Sig1R promotes the formation of FDM-induced B1-integrin / hERG complexes. (**a**) Representative images showing membrane distribution of GFP-tagged proteins (green) and β1-integrin (red) staining and colocalization areas (yellow). K562 sh RD and sh Sig1R cells were transiently transfected with a GFP-tagged hERG1a or Sig1R. 48h later, cells were harvested and plated 30 min on either uncoated- or FDM-coated glass coverslips. After fixation, cells were labeled with an Alexafluor 594-labelled antibody directed against an extracellular part of the β1-integrin subunit. Colocalization between GFP-tagged protein and β1 integrin is expressed as the increase of the Pearson's coefficient in FDM when compared to uncoated condition (right panel; n = 12 to 20 cells from 2 independent experiments; *** $p < 0.001$). (**b**) Flow cytometry-based FRET assay for the identification of hERG/β1-integrin complexes at the plasma membrane. (**c**) Left panel: representative FACS-contour plots showing the amount of FRET-positive cells in total K562 sh RD or sh Sig1R cells populations, plated for 30 min on uncoated or FDM-coated dishes. Right panel: Corresponding histograms representing the mean (± SEM) of FRET-positive cells in each condition. (6 to 7 independent experiments; * $p < 0.05$).

Figure 3. Sig1R silencing inhibits FDM-triggered AKT signaling pathway. Left panel: Total protein extracts from K562 sh RD or sh Sig1R cells plated for 30 min on uncoated or FDM-coated dishes in presence or absence of hERG inhibitor, E-4031 (50μM), were separated on SDS-PAGE and immunoblotted with antiphosphorylated-AKT (ser 473), AKT and Sig1R antibodies (representative immunoblot from 6 independent experiments). Right panel: Corresponding histogram showing the phosphorylation of AKT ratio for each condition (phosphorylated versus total AKT; values are mean ± SEM, * $p < 0.05$; ** $p < 0.01$).

Figure 4. FDM stimulates cell migration and actin organization in a Sig1R manner. (**a**) Plots representing 3h-cell tracking. K562 sh RD or sh Sig1R cells were plated on either uncoated or FDM-coated dishes in the absence (FDM) or in the presence of E-4031 (50 μM)

or LY 294002 (10 µM). (**b**) Corresponding histograms quantifying motility parameters. Left panel: total length traveled per cell. Right panel: Euclidian distance traveled per cell. Values are mean ± SEM recapitulating the tracking of n= 202-410 cells (from 2 or 3 independent experiments; t-test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (**c**) K562 sh RD and sh Sig1R cells were plated on either uncoated or FDM-coated glasses, in the absence or presence of E-4031 (50 µM) or LY294002 (10 µM). After 3h, cells were fixed, permeabilized and stained for actin using FITC-conjugated phalloidin. Representative images showing spike actin organization (green labeling) in each condition. Right panel: corresponding histogram representing the mean (± SEM) of actin spikes per cells in each condition (n = 15 to 40 cells from 2 independent experiments, ** $p < 0.01$; *** $p < 0.001$).

Figure 5. Sig1R drives CML invasiveness potency by upregulating VEGF secretion, transendothelial migration, angiogenesis and extravasation. (**a**) scheme representing the protocol of zebrafish xenograft assay for migration. (**b**) Sig1R-dependent *in vivo* invasion of K562 from the yolk sac of zebrafish embryos 48h after engraftment (see material and methods). Left panel: representative images of zebrafish embryos negative (no K562 cells outside the yolk sac) or positive for K562 invasion (more than 3 cells outside the yolk sac). Right panel: histogram showing the percentage of positive zebrafish embryos for K562 sh RD or sh Sig1R cell invasiveness from a total of embryos indicated in the figure for each conditions. (values are mean ± SEM of 3 to 5 independent experiments; * $p < 0.05$). (**c**) VEGF secretion evaluated in K562 sh RD or sh Sig1R cells in the absence (Control) or the presence of E-4031 (40 µM) (values are mean ± SEM of 3 independent experiments; *** $p < 0.001$). (**d**) scheme representing the protocol of xenograft in zebrafish for angiogenesis assay. (**e**) Tumour-induced angiogenesis was evaluated in zebrafish embryos (see materials and methods). K562 sh RD or sh Sig1R cells were injected in the perivitellin space of zebrafish embryos. 24h later, vessels were visualized. Left panel: representative images of a zebrafish negative (left) and a zebrafish positive (right) for K562-induced angiogenesis. An esterisk indicates the injection area). Right panel: histograms representing the percentage of positive and negative zebrafish embryos from a total number stated in the figure for each condition. (t-test. * $p < 0.05$). (**f**) Histograms showing the percentage of K562 sh RD or sh Sig1R cells migration through a primary endothelial (HUVEC) cell monolayer seeded onto a Boyden chamber. Values are mean ± S.E.M. (n= 4; * $p < 0.05$). (**g**) scheme representing the protocole for pulmonary extravasation of K562 cells. (**h**) Pulmonary extravasation of K562 cells injected in NOD-SCID Hairless mice tail vein. Left panel: representative images showing localization of K562 sh RD or sh Sig1R cells in lung at 30 mn and 24h post-injection.

Right panel: Corresponding histogram of the number of K562 cells per field in lung at 24h post-injection. (Values are mean ± SEM from 3 independent experiments. * $p < 0.05$).

Supplementary figure 1. FDM induces a stable increase in hERG current in a Sig1R-dependent manner. Histograms showing hERG current density recorded in sh RD and sh Sig1R K562 cells plated for 15, 30 or 180 min on uncoated or FDM-coated dishes. (n = 6-15 cells per conditions). (* $p < 0.05$; ** $p < 0.01$; N.S.: Non significant)

Supplementary figure 2. FDM alters neither hERG maturation nor β1 integrin subunit surface expression. K562 sh RD and sh SigR were plated 30 min on uncoated or FDM-coated dishes. (**a**) Immunoblot showing hERG, tubulin and Sig1R proteins expression in each condition. Tubulin was used as a loading control. (**b**) Corresponding histograms quantifying relative hERG expression and hERG 1b maturation (upper and bottom panels, respectively). Values are mean ± S.E.M. (n= 7; N.S. non-significant). (**c**) The plasmalemmal fraction of β1 integrin subunit was labeled with an antibody directed against an extracellular epitope of β1 integrin. The percentages of β1 integrin-positive K562 sh RD or sh Sig1R cells plated on uncoated or FDM-coated dishes were determined by flow cytometry. Left panel: Representative histograms showing β1 integrin surface expression in each condition. Right panel: Corresponding histograms representing the mean ± SEM of 7 independent experiments (N.S. Non-significant).

Supplementary figure 3. Sig1R is associated to β1 integrin and promotes β1 integrin / hERG association

Immunoprecipitation of β1-integrin subunit in HEK293 cells stably expressing hERG1a and/or cmyc-tagged-Sig1R or silenced for Sig1R expression (sh Sig1R). Immunoblots were performed using anti-hERG or anti-Sig1R antibodies (representative immunoblot from 5 independent experiments).

Supplementary figure 4. FDM or Sig1R silencing does not affect β1 integrin surface expression.

Plasmalemmal fraction of β1 integrin subunit was labeled with an antibody directed against an extracellular epitope of β1 integrin. The percentages of β1 integrin-positive K562 sh RD or sh Sig1R cells plated on uncoated or FDM-coated dishes were determined by flow cytometry. Left panel: Representative histograms showing β1 integrin surface expression in each condition. Right panel: Corresponding histograms representing the mean ± SEM of 7 independent experiments (N.S. Non-significant).

Supplementary figure 5. Sig1R promotes hERG- and AKT-dependent FDM-induced cell spreading. CFSE-labeled K562 sh RD and sh Sig1R were plated for 3h on either uncoated or FDM-coated glasses in absence or presence of E-4031 (50 μM) or LY294002 (10 μM). Cells were fixed and analyzed for their circularity and classified as circular or not circular as a function of the circularity coefficient (> 0.5 or < 0.5, respectively). (**a**) Representative images of K562 cells 3h after plating on FDM (corresponding circularity coefficient is indicated for each case). (**b**) Left panel: distribution of circularity coefficients of K562 sh RD and sh Sig1R plated 3h on FDM-coated glasses. Right panel: evolution of the percentage of not-circular cells as a function of time in K562 sh RD or sh Sig1R plated on FDM-coated glasses. (**c**) Histogram representing the percentage of non-circular cells in K562 sh RD or sh Sig1R populations plated 3h on uncoated or FDM-coated glasses. Experiments were performed in absence or the presence of E-4031 (50 μM) or LY294002 (10 μM) (n = 3 to 5 independent experiments for a total of 265 to 918 cells. * $p < 0.05$; ** $p < 0.01$).

Supplementary Figure 6. Sig1R is over expressed in human leukemia and CRC

(**a**) Intensity of Sig1R mRNA expression in human CML(Affer et al., 2011), AML(Andersson et al., 2007) and CRC(Hong et al., 2010) patients. mRNA expression values in cancer patients (blue boxes) are compared to corresponding normal tissues (white boxes) ($p = 0.0028$, $p = 8 * 10^{-8}$ and $p= 1.2 * 10^{-9}$, respectively). (**b**) Since HCT-116 CRC cell migration and VEGF secretion are both driven by hERG expression(Crociani et al., 2014b; Lastraioli et al., 2004), we generated Control (sh RD) and Sig1R-silenced (sh Sig1R) HCT-116 cell lines. The blots show the expression of Sig1R in both cell lines. (c) Migrating control (sh RD) and Sig1R-silenced (sh Sig1R) HCT-116 colorectal cells in the absence (Control) or the presence of E-4031 (30 μM) in Boyden chamber assay as previously described (Lastraioli et al., 2004) (left panel). Values are presented in the corresponding histogram (right panel) (Mean ± SEM of 3 to 5 independent experiments, * $p < 0.05$). (**d**) VEGF secretion evaluated in HCT-116 sh RD or sh Sig1R cells in the absence (Control) or the presence of E-4031 (40 μM). Values are

mean ± SEM (n = 3; * $p < 0.05$). (**e**) protocol used for the evaluation of HCT-116 cell invasive potency in zebrafish. (**f**) Sig1R-dependent *in vivo* invasion of HCT-116 cells from the yolk sac of zebrafish embryos 48h after engraftment (see material and methods). Left panel: representative images of negative (no cells outside the yolk sac) or positive (more than 3 cells outside the yolk sac) zebrafish embryos for HCT-116 cell invasion. Right panel: histogram showing the percentage of positive zebrafish embryos for K562 sh RD or sh Sig1R cell invasiveness from a total of embryos indicated in the figure for each conditions (values are mean ± SEM of 2 independent experiments; exact Fischer's test; *** $p < 0.001$).

Materials and methods

Chemicals, antibodies and reagents. Unless otherwise stated, all cell culture media, supplements and antibiotics were purchased from Gibco BRL (Life technologies, Gaithersburg, MD, USA). For flow cytometry (FC), immunocytochemistry (IC), immunoprecipitation (IP) or western blot (WB), we used the following antibodies at the indicated concentrations: Rabbit anti-hERG pan (WB : 1/2000) (Enzo life sciences, Farmingdale, NY, USA); Mouse anti-hERG extracellular (FC : 1/100) (Enzo Life Sciences, Farmingdale, NY, USA); Mouse anti-B1 integrin (WB : 1/1000 ; IF : 1/100 ; IP : 2 µg / mL) (Biolegend, San Diego, CA, USA); Mouse Alexafluor488-conjugated anti-B1 integrin (FC : 1/100) (Biolegend, San Diego, CA, USA); Mouse anti-tubulin (WB : 1/50000) (Sigma-aldrich, Saint-Louis, MO, USA); Mouse anti-Sig1R (WB : 1/1000) (Santa Cruz Biotechnology, Dallas, TX, USA); Rabbit anti-Akt total (WB : 1/1000) (Cell signaling, Danvers, MA, USA); Rabbit anti-phospho Akt (WB : 1/1000) (Cell signaling, Danvers, MA, USA); Swine HRP-conjugated anti-rabbit (WB : 1/2000) (Dako, Glostrup, Denmark); Goat HRP-conjugated anti-mouse (WB : 1/5000; Dako, Glostrup, Denmark); Goat Alexafluor594-conjugated anti-mouse (IF : 1/400 ; FC : 1/500; Invitrogen, Carlsbad, NM, USA); Donkey Alexafluor647-conjugated anti-rabbit (IF : 1/400) (Invitrogen, Carlsbad, NM, USA); Rabbit anti-eGFP (FC : 1/5000 ; WB : 1/10000) (Abcam, Cambridge, UK).

Cell culture. K562, NIH3T3 and HEK cell lines were cultured as previously described (Crottes et al., 2011). Briefly, the K562 cell line was obtained from Dr S. Brown (Cambridge, UK) and cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. NIH3T3 and HEK 293 cells were cultured in DMEM medium supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin.

shRNA transduction. Control or Sig1R-silenced cells were established as previously described (Crottes et al., 2011). Briefly, lentiviral particles were obtained from Sigma "MISSION shRNA Lentiviral transduction particles" (Sigma-Aldrich, St Louis, MO, USA). Day 1, K562 or HEK stably expressed hERG1a cells were plated in a 6-wells plate at a density of 20 000 cells/well in complete medium. Day 2, medium was removed and cells were incubated in complete medium containing 8 µg/ml of hexadimethrin bromide (Sigma-aldrich, Saint-Louis, MO, USA) and transduced at a MOI of 5. Clones SHC002V (non-target shRNA) and SHVRC-TRCN0000061011 (shSig1R targeted) were used for transduction. Day 3, a new transduction round was applied. Day 6, puromycin (0.5 mg/ml) was added in fresh medium to start selection of transduced cells.

Transient transfection of K562. K562 expressing eGFP-hERG or eGFP-Sig1R was established using lipofectamine 2000 following the protocol supplied by the manufacturer (Invitrogen, Carlsbad, NM, USA). Briefly, K562 cells were plated in a 6-well plate at a density of 5.10^5 cells / well in complete medium. 2 hours later, DNA plasmids encoding eGFP-hERG or eGFP-Sig1R and lipofectamine were diluted in a RPMI-free medium (0.5 mL). Cells were harvested and used 48h later.

hERG-transduced HEK 293. A HEK cell line stably expressing hERG channel was established as previously described (Crottes et al., 2011). Briefly, the coding sequence of hERG1 (kind gift of Dr. G. Robertson, Wisconsin University, USA) was subcloned into the mammalian expression vector pPRIhygro to generate pPRIhygro-hERG1. pPRIhygro is derived from pPRIpu[23] where puromycin resistance gene was exchanged for hygromycin resistance gene (sequences of pPRIpu and pPRIhygro are available on request). Highly pure recombinant plasmids were obtained by anion-exchange chromatography (NucleobondAx, Macherey-Nagel, Duren, Germany) and were used to stably transduce HEK 293 cells. For transduction experiments, HEK293 cells were seeded at 30-40% density in 100 mm dishes in DMEM supplemented with 10 % FCS. To generate retroviruses, 293T cells were transfected the following day with 10 µg of an empty pPRIhygro plasmid or the pPRIhygro-hERG1 construct, and with 5 µg of pCMV-VSVG and 5 µg of pCMV-gag-pol plasmids using the classic calcium phosphate transfection technique. 6 h after transfection, cells were washed and fresh medium was added. Replication-defective retroviruses were recovered in the culture medium between 24 h and 72 h post-transfection. These retroviral supernatants were

filtered through sterile 0.45 µm filters, then added directly to HEK 293 cells in the presence of 4 µg/ml hexadimethrin bromide to enhance retroviral transduction efficiency. On day 6, hygromycin (100ng/ml) was added in fresh medium to start selection of transduced cells. Western blot analyses were performed to check correct expression of hERG1.

Cmyc Sig1R transfection. Similarly, cmyc-Sig1R cDNA (cmyc-tag added in phase at the N-terminal part of the protein) (7) was subcloned in pPRIPu vector. 10 µg of pPRIPu-cmyc-Sig1R cDNA was transfected in HEK wild type or stably expressing hERG1 using the classic calcium phosphate transfection technique. Retention of stable HEK cell expressing hERG/cmycSig1R and cmyc-Sig1R was achieved by puromycin selection.

Generation of fibroblast-derived matrices (FDM). The protocol used was the same as described by Beacham et al (Beacham et al., 2007). Briefly, NIH3T3 cells were cultured at confluence during 7 days with DMEM medium supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin and ascorbic acid 50 µg/mL. Then, cells were removed by using PBS 1X Triton X100 0.1%, Ethanolamin 20 mM. Matrices were then rinsed using PBS 1X supplemented with $CaCl_2$ and $MgCl_2$ (1 mM each) before use.

Patch-clamp experiments. K562 cells were prepared as previously described (Crottes et al., 2011; Renaudo et al., 2004). The external solution was: 45 mM KCl, 90 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes (pH adjusted to 7.4 with HCl, 285 mosm/l). Soft glass patch electrodes (Brand, Wertheim, Germany) were made on a horizontal pipette puller (P-97, Sutter Instrument Co., Novato, CA, USA) to achieve a final resistance ranging from 3 to 5 MΩ. The internal solution was: 130 mM K-aspartate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM Hepes, 2 mM ATP and 100 µM GTP (pH adjusted to 7.2 with KOH, 290 mosm/l). Electrical signals were amplified with an Axopatch 200B amplifier (Molecular Device, Sunnyvale, CA, USA) and acquired with a DIGIDATA 1440 interface and pCLAMP 10.2 software (Molecular Device, Sunnyvale, CA, USA). K^+ currents were recorded at a 10 kHz-sampling frequency and filtered at 2 kHz.

Immunoprecipitation and Western blot experiments. For immunoprecipitation, cells were then lysed in 1ml of lyse buffer (10mM Tris, 1mM EDTA pH 8.00, 1mM phenylmethylsulfonyl

fluoride (PMSF), scrapped, and homogenized with a Potter-Elvehjem Pestle. Homogenates were centrifuged at 500g for 1min to remove large cell fragments. Supernatants were collected and centrifuged for 1 hour at 10,000g. Pellets containing plasma membranes were homogenized in ice cooled IP buffer (150nM NaCl, 50mM Tris, pH 8.00; 0,5mM EDTA). Protein suspension were immunoprecipitated using myctag (dilution 1:300) and CD29 (dilution 1:200) antibodies, respectively, in IP-buffer 1% dodecyl-β-D-maltoside (DDM). Samples were incubated with 2% BSA saturated anti-mouse IgG (whole molecule)-agarose beads overnight. Next day, beads were washed in PBS 0.5% DMM for 5 min and then washed four times for 10 min in PBS 0.1% DMM. Total protein extracts were obtained by using RIPA buffer containing 0.5 mM Pefabloc. The homogenate was centrifuged at (12000 rpm) for 10 in at 4°C. The pellet was discarded and supernatant was solubilized in a suitable volume of lysis buffer. Finally proteins were separated on SDS-PAGE electrophoresis. Then, proteins were blotted onto PVDF (Millipore, Molsheim, France) membranes, using a BioRad semi-dry transfer system (Whatman, Göttingen, Germany). Membranes were blocked in non-fat milk 5% in TBS Tween 0.1% during 1h and then incubated overnight at 4°C with primary antibodies at the appropriate dilution. Membranes were washed in TBS Tween 0.1% supplemented with non-fat milk 1%. Peroxidase-coupled secondary antibodies were applied at the appropriate dilution in TBST supplemented with non-fat milk 1%. Then, membranes were rinsed using TBST. Signals were visualized using ECL reagent (Pierce, Rockford, IL, USA) on a Fusion FX-7 image acquisition system (Vilber Lourmat, Torcy, France). Densitometric analysis of the data were performed with Image J analysis software (NIH, Bethesda, MD, USA) and the results were corrected for protein loading by normalization for α-tubulin or actin expression.

Flow cytometry and flow cytometry-based FRET assay. K562 cells were fixed in PBS 1X PFA 4% for 10 min and were successively incubated for 30 minutes at 4°C in a solution of PBS / FBS 3% / EDTA 2mM containing the primary antibody at the appropriate dilution. For unconjugated-primary antibodies, cells were washed and incubated in a solution containing the fluorophore-conjugated secondary antibodies. Cells were analysed with a BD LSR Fortessa and FACS Diva software (Becton Dickinson, Franklin Lakes, NJ, USA). The use of an appropriate isotypic control allowed eliminating non-specific signal in the population. The methodology of flow cytometry-based assay was previously described by Banning et al. (Banning et al., 2010). Briefly, the donor protein was stained with Alexafluor488-conjugated antibody and the acceptor protein was stained with Alexafluor594-conjugated antibody. To measure Alexafluor488 and FRET signals, cells were excited with a 488 nm laser and fluorescence were collected respectively with a LP mirror 505 nm and a BP filter 530/30 nm,

or with a LP mirror 600 nm and a BP filter 610/20 nm. To measure AlexaFluor594 signals, cells were excited with a 594 nm laser and fluorescence were collected with a LP mirror 600 nm and a BP filter 610/20 nm.

Immunofluorescence and confocal analysis. For colocalization experiments, cells were plated on uncoated or FDM-coated glasses. After 30 min, cells were fixed with PBS 1X PFA 4% and then subsequently incubated during 1h at 4°C with mouse anti-B1 integrin and goat Alexafluor594-conjugated anti-mouse. For actin cytoskeleton visualization, cells were plated 3h on different substrates, before to be subsequently fixed with PBS 1X PFA 4%, permeabilized with PBS 1X Triton X100 0.1% (Sigma-aldrich, Saint-Louis, MO, USA), blocked with PBS BSA 5% and incubated with FITC-phalloidin 1X (Sigma-aldrich, Saint-Louis, MO, USA). Cell nucleus were stained with Hoescht 33342 and glasses were mounted with mounting medium (Fluka, Saint-Louis, MO, USA) onto coverslips. Acquisitions were performed on a confocal spinning disk microscope (Olympus, Southend-on-Sea, UK) equipped with an ANDOR camera and a 100X oil-objective.

Analysis of cell spreading. Cells were stained with CFSE (5 µM) the day prior the experiment. Then, cells were plated on uncoated or FDM-coated dishes. At different time points, cells were fixed with PBS1X PFA 4% and slides were mounted with mounting medium (Fluka, Saint-Louis, MO, USA) onto cover glasses. Acquisitions were performed on a confocal spinning disk microscope (Olympus, Southend-on-Sea, UK) at 60X objective. Circularity coefficient were measured with Image J software (NIH, Bethesda, USA).

Time-lapse motility assay. Cells were stained with 5 µM of CFSE (Invitrogen, Carlsbad, NM, USA) and plated on a 6-well plate. 1 h later, cell motility was acquired using a Nikon Ti Eclipse microscope for 3h using thermostated chamber and CO_2 pressure. At least 130 individual cells per condition and per experiment were tracked with Fiji/MTracks2 software (NIH, Bethesda, MD, USA).

Transendothelial migration. HUVEC primary endothelial cells were grown in EGM-2 and were cultured on the upper membrane surface of a Boyden chamber overnight to generate a confluent monolayer. K562 cells were seeded into the HUVEC-coated Boyden chamber membrane. Cells were seeded into non-coated plates as a suspension control. Cells were

incubated at 37°C, CO_2 5% during 24h. Then, migrated cells in the lower chamber were harvested and counted in triplicate by using BD LSR Fortessa (HighTroughput System) (Becton Dickinson, Franklin Lakes, NJ, USA).

Human cancer dataset analysis. After a first survey performed using OncoMine (Rhodes et al., 2007), we analyzed four datasets corresponding to colorectal cancer (CRC) – 82 donors, acute myeloid leukemia (AML) - 29 donors, and chronic myeloid leukemia (CML) - 12 donors. The different datasets correspond to the following GEO(Barrett et al., 2013) accession number respectively: GSE9348(Hong et al., 2010), GSE7186(Andersson et al., 2007), GSE24739(Affer et al., 2011). The normalized data were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/geo/) and their visualization was performed using the ggplot2 package in R (http://cran.r-project.org/web/packages/ggplot2/index.html). For each dataset, a Mann-Whitney test was used to measure a significant ratio between cancer and normal intensity. Each *p*-value is indicated in the figure legend.

VEGF secretion assay (ELISA). K562 cells were seeded onto fibronectin-coated 12 wells plates ($3x10^5$ cells per well), in RPMI medium depleted of FBS, and incubated for 48 hours. VEGF concentration was measured in cell culture supernatants using a standard enzyme-linked immunosorbent assay (ELISA) protocol (human VEGF-A ELISA kit, Thermo Scientific, France). VEGF concentrations were normalized by the corresponding cell number. The role of hERG on VEGF secretion was assessed by adding 40 µM E4031 in the cell incubation medium. Each experiment was performed in triplicate.

Zebrafish maintenance and zebrafish invasion assays. Zebrafish (*Danio rerio*) AB, from the Zebrafish International Resource Centre, was maintained in re-circulating tanks according to standard procedures ('The zebrafish handbook: a laboratory use of zebrafish, Brachydanio rerio'). Adult fish were maintained at 261C, with a light/dark cycle of 14/10 h and were fed twice daily, once with dry flake food (PRODAC, Malaga, Spain) and once with live *Artemia* (MC 450, IVE AQUACULTURE, INVE Aquaculture, Vigo, Spain). Zebrafish embryos were maintained in egg water at 28.51C, fed during 5 days with NOVO TOM and with live *Artemia* at 11 days of life. The experiments performed comply with the Guidelines of the European Union Council for animal experimentation (86/609/EU) and were approved by the Bioethical Committee of the University Hospital Virgen de la Arrixaca. K562 were stained with the vital cell tracker red fluorescent CM-Dil (Vibrant, Invitrogen, Carlsbad, NM, USA)

centrifuged, resuspended in 67% of DPBS, 5 fetal calf serum, 0.05% Phenol Red and injected into the yolk sac of dechorionated zebrafish embryos using a method described previouslt(Marques et al., 2009). Briefly 100–150 labelled cells in 4 nl were injected into the yolk sac of zebrafish embryos using a manual injector (Narishige, East Meadow (Long Island), NY, USA). Fish with fluorescent cells outside the implantation area at 2h were excluded from further analysis. All other fish were incubated at 35°C for 48h and analyzed with a SteReo Lumar.V12 stereomicroscope with an AxioCam MR5 camera (Carl Zeiss, Thornwood, NY, USA). Evaluation criteria for invasion were that at least three cells had to be identified outside of the yolk.

Tumour cell zebrafish embryo angiogenic assay. Experiments were performed as already described with minor modifications (Nicoli and Presta, 2007). Briefly, a zebrafish (*Danio rerio*) breeding colony (wild-type AB strain) was maintained at 28°C at the Zebrafish Facilities of the University of Brescia. Embryos were collected by natural spawning and staged according to Kimmel et al. (Kimmel et al., 1995). Dechorionated embryos were anesthetized at 48 h post fertilization (hpf) with 0.04 mg/mL of tricaine (Sigma-aldrich, Saint-Louis, MO, USA) and injected into the perivitelline space, between the yolk and the periderm in the proximity of developing SIVs, with 400 cells per embryo resuspended in 4 nl of 0.5% sodium alginate (Sigma-aldrich, Saint-Louis, MO, USA) using a Femtojet microinjector system (Eppendorf, Hamburg, GER). Embryos injected with 4 nl of 0.5% sodium alginate were used as controls. After tumour cell or vehicle injection, embryos were sequentially incubated with 5 mM $CaCl_2$ for 30 minutes at room temperature and then for 24 hours at 33°C in fish water. Next, embryos were fixed in 4% paraformaldehyde for 2 h at room temperature and stained for endogenous alkaline phosphatase activity to visualize the angiogenic response elicited by cell grafts(Serbedzija et al., 1999). For that purpose, embryos were mounted in agarose-coated Petri dishes and photographed under an epifluorescence Leica MZ16 F stereomicroscope equipped with a QIClick digital CCD camera and Qcapture Pro 6.0 software (Qimaging, Surrey, CA). Embryos were scored for a positive angiogenic response defined as the presence of a new alkaline phosphatase-positive microvasculature projecting from the sub-intestinal vessel plexus of the embryo and infiltrating the tumour graft(Nicoli and Presta, 2007).

Lung extravasation assay. K562 transfected with shRD or shSig1R were fluorescently labeled with CellTracker™ Green. 10^7 cells (Sig1R depleted or control cells) were injected into the tail vein of Hairless NOD.SCID (Harlan Laboratories) mice sacrificed 30 min or 24h later.

Lungs were harvested for analysis with a 5X Zeiss Inverted scope. Lungs harvested at 24h were perfused to eliminate cells remaining in blood system and allow the detection of evading cells.

Statistics. Unless otherwise stated, statistical differences between control and sh Sig1R cell populations were assayed by Mann-Whitney test. In all cases, a difference with a p value less than 0.05 was considered as significant.

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Author contributions

D.C. designed and performed patch-clamp, cell biology *in vitro* experiments and participated in writing the manuscript. M.L.C. supervised zebrafish invasion experiments. F.A.P. and D.C. performed zebrafish invasion assay. F.B. established all sh cell lines and performed co-immunoprecipitation experiments. S.M. performed VEGF measurements and participated to the redaction. P.M. built initial constructs and validated them *in vitro*. M.P. and G.G. designed and performed i*n vivo* angiogenesis assays. D.C. designed and performed *in vitro* transendothelial migration experiments. S.T.D. and M.T. designed and performed mice extravasation experiments. A.L. contributed to FACS experimental design. A.P. performed genomic dataset statistical analysis. B.P. was in charge of cell culture maintenance and

performed so western blot experiments. H.G. participated to data analysis. O.S. supervised the study, designed experiments, contributed to data analysis and wrote the manuscript.

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Figure 1

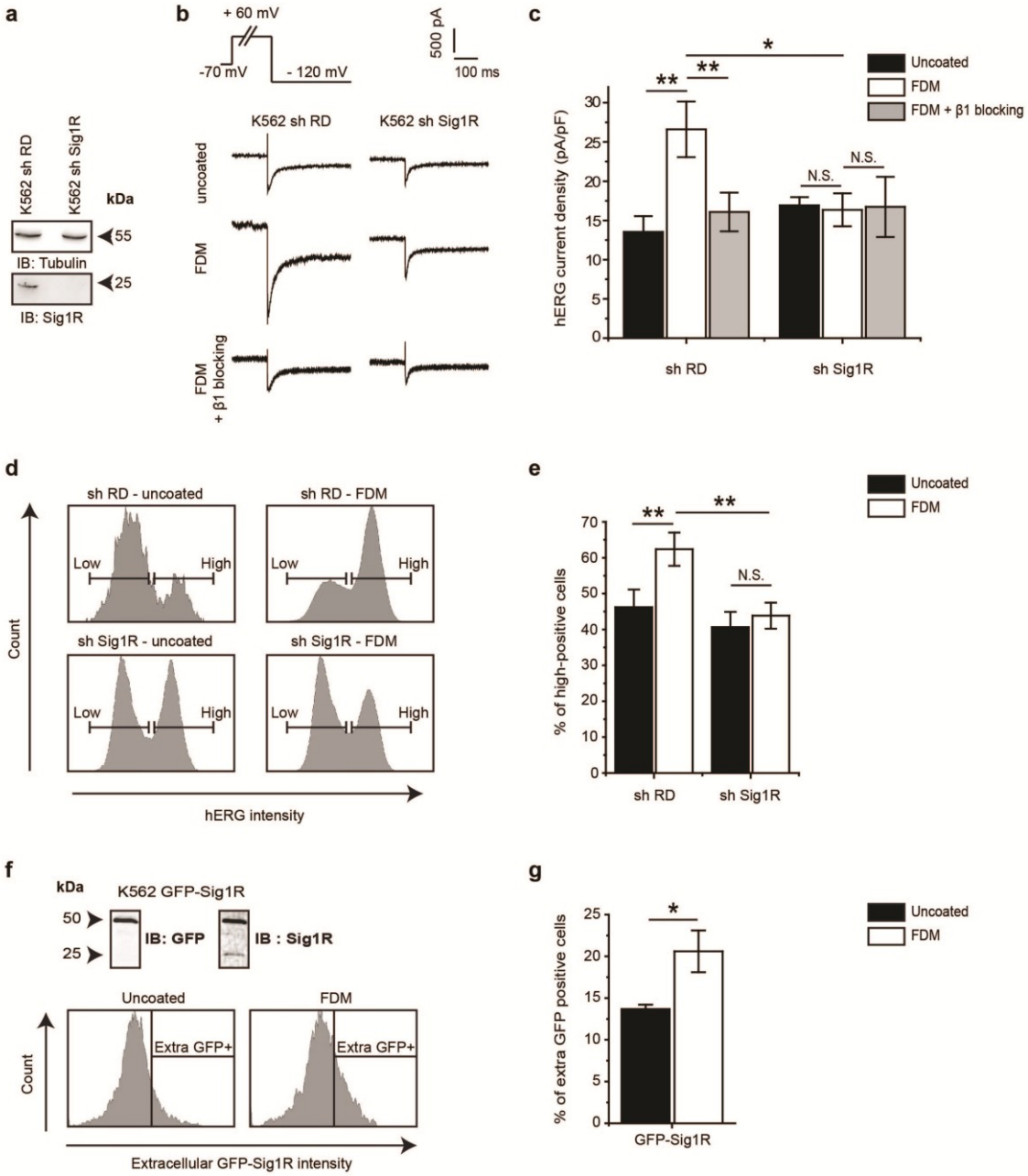

a
K562 sh RD
K562 sh Sig1R
kDa
55
IB: Tubulin
25
IB: Sig1R
b
+ 60 mV
-70 mV
- 120 mV
500 pA
100 ms
K562 sh RD
K562 sh Sig1R
uncoated
FDM
FDM + β1 blocking
c
Uncoated
FDM
FDM + β1 blocking
hERG current density (pA/pF)
N.S.
sh RD
sh Sig1R
d
sh RD - uncoated
sh RD - FDM
sh Sig1R - uncoated
sh Sig1R - FDM
Low
High
Count
hERG intensity
e
Uncoated
FDM
% of high-positive cells
N.S.
sh RD
sh Sig1R
f
kDa
K562 GFP-Sig1R
50
25
IB: GFP
IB : Sig1R
Uncoated
FDM
Extra GFP+
Count
Extracellular GFP-Sig1R intensity
g
Uncoated
FDM
% of extra GFP positive cells
GFP-Sig1R

Figure 2

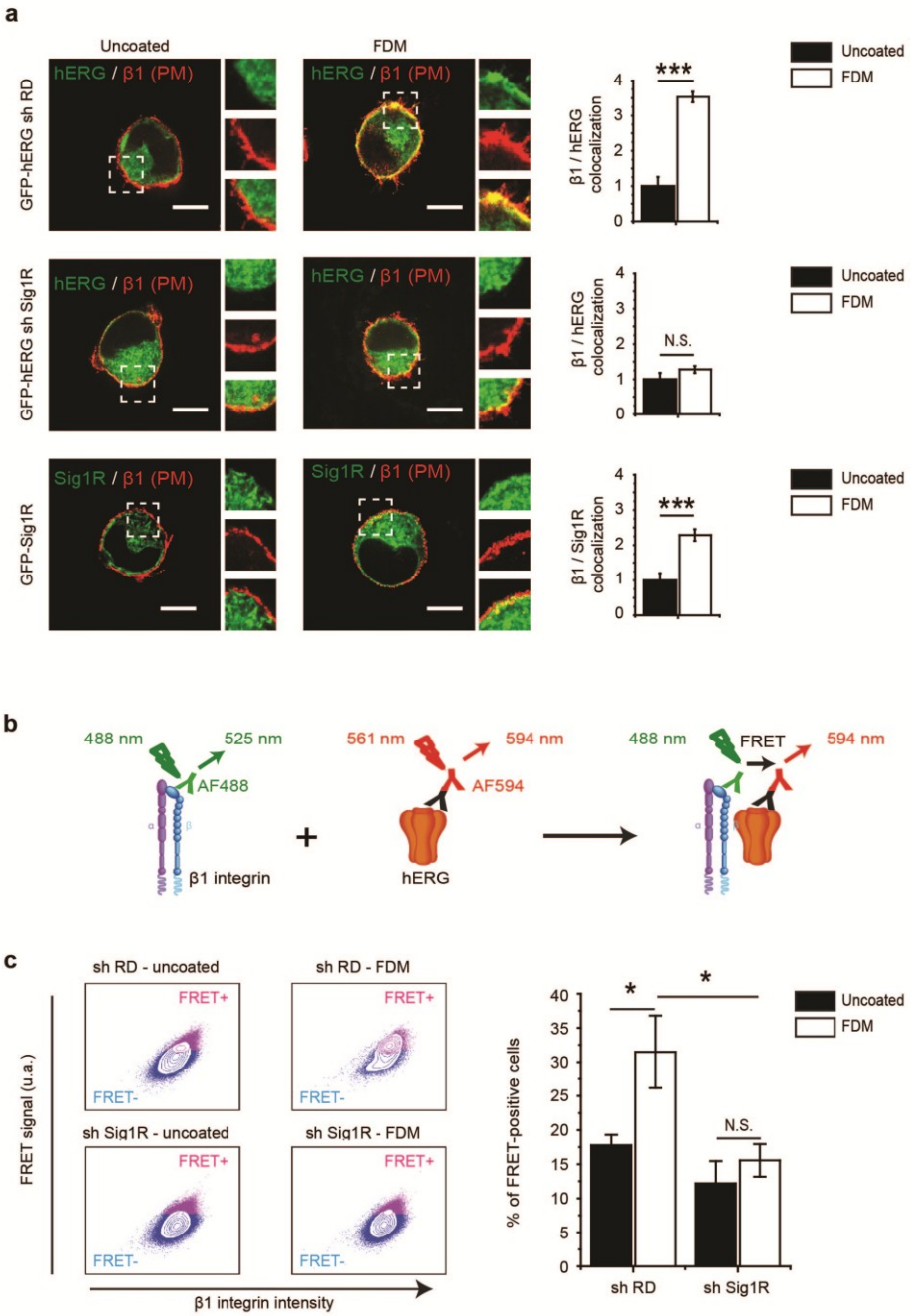

a
Uncoated
FDM
GFP-hERG sh RD
hERG / β1 (PM)
β1 / hERG colocalization
***
Uncoated
FDM
GFP-hERG sh Sig1R
N.S.
GFP-Sig1R
Sig1R / β1 (PM)
β1 / Sig1R colocalization
b
488 nm
525 nm
AF488
β1 integrin
561 nm
594 nm
AF594
hERG
FRET
c
sh RD - uncoated
sh RD - FDM
sh Sig1R - uncoated
sh Sig1R - FDM
FRET+
FRET-
FRET signal (u.a.)
β1 integrin intensity
% of FRET-positive cells
sh RD
sh Sig1R

Figure 3

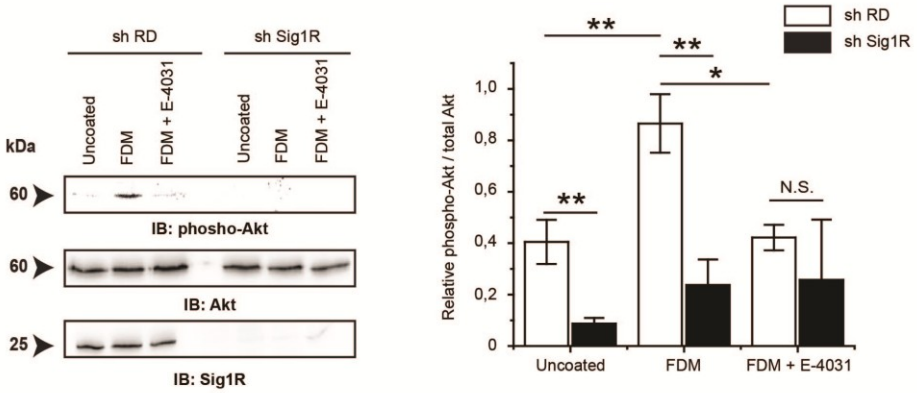
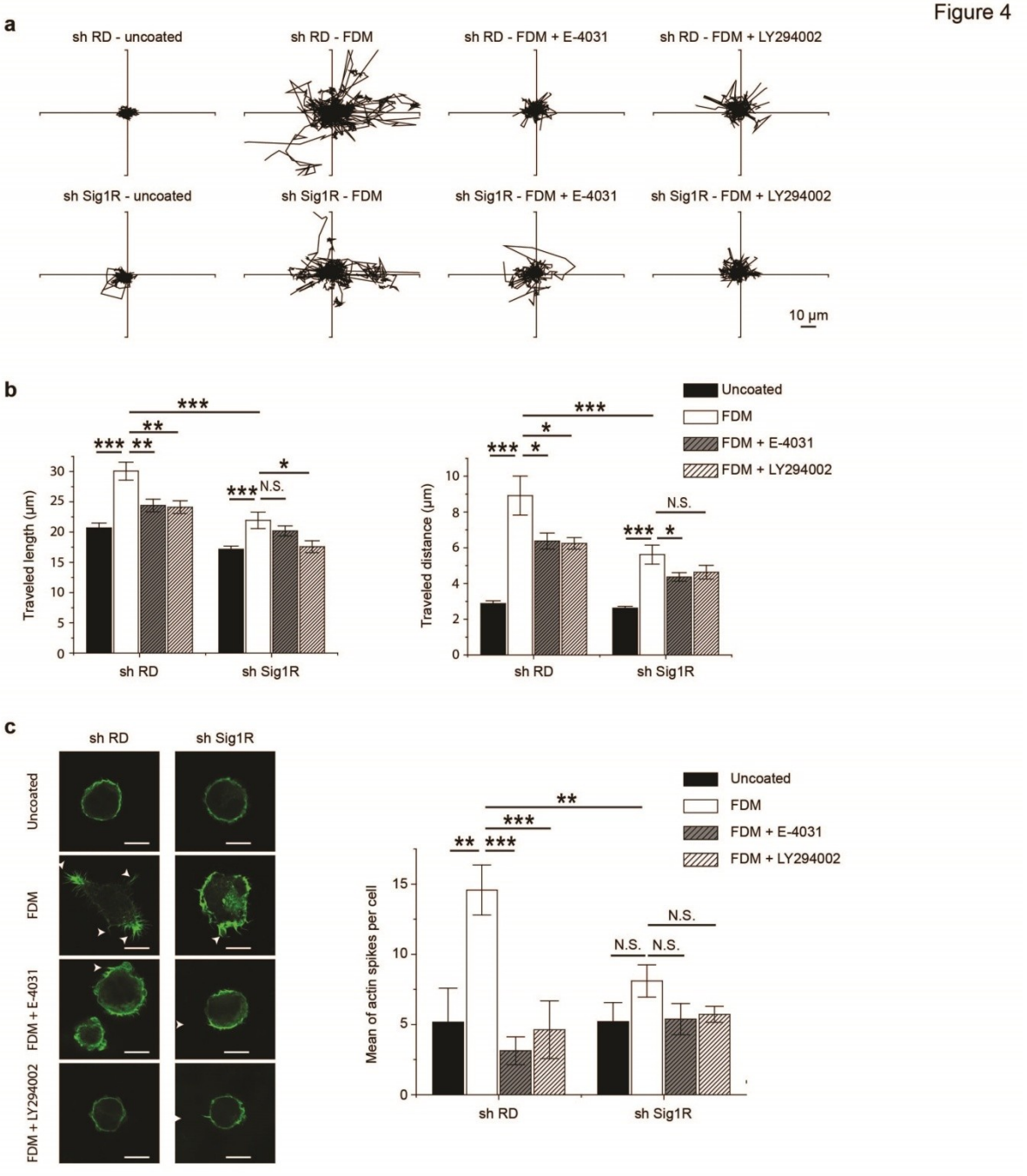
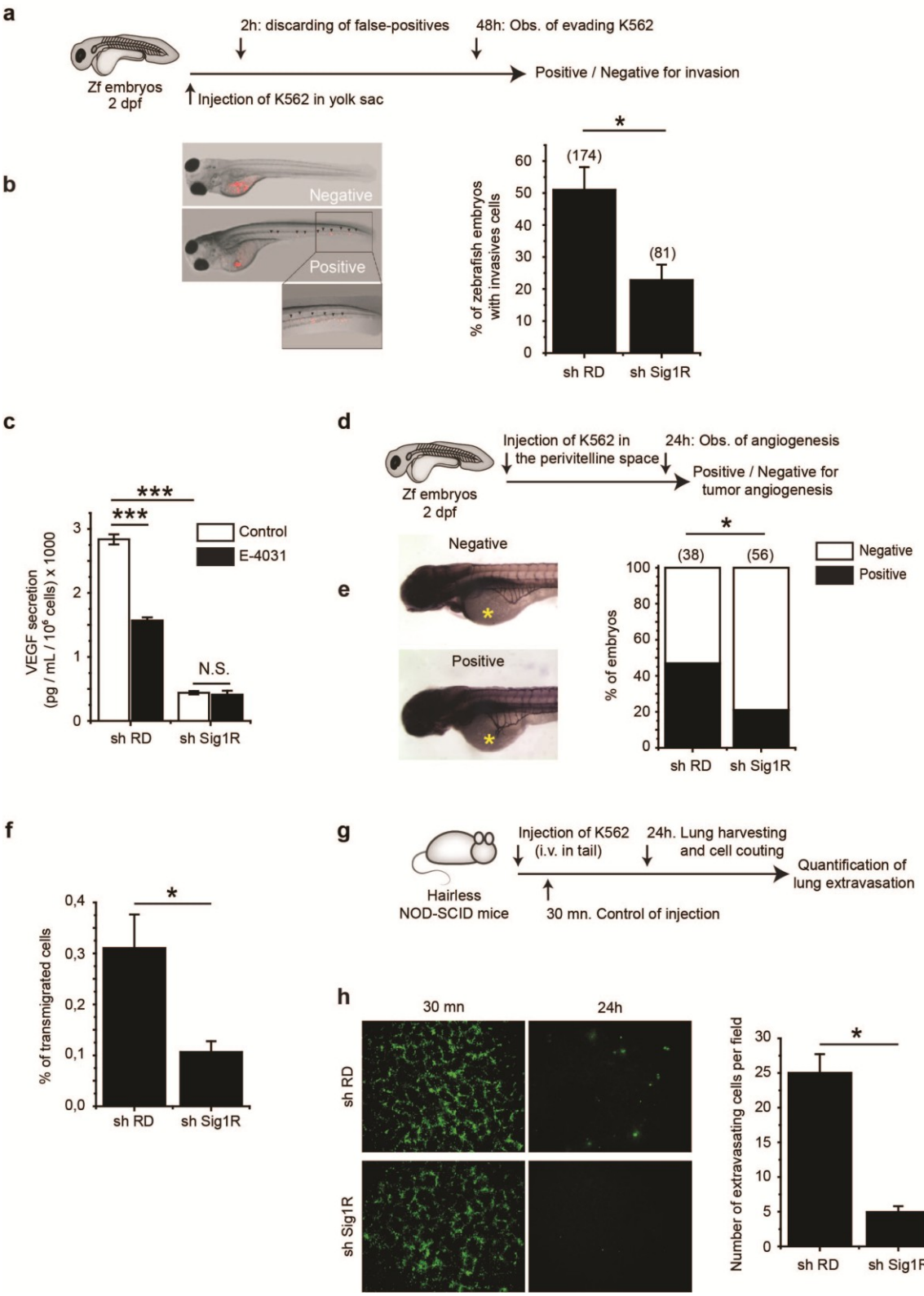
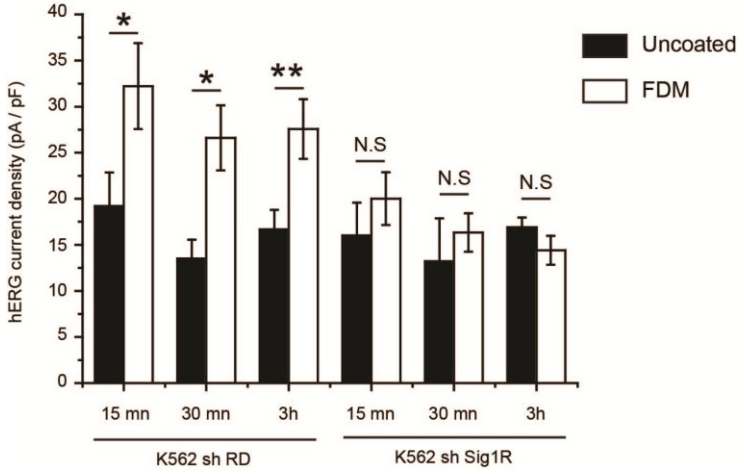

sh RD
sh Sig1R
Uncoated
FDM
FDM + E-4031
Uncoated
FDM
FDM + E-4031
kDa
60
IB: phosho-Akt
60
IB: Akt
25
IB: Sig1R
sh RD
sh Sig1R
Relative phospho-Akt / total Akt
1,0
0,8
0,6
0,4
0,2
0
**
**
*
**
N.S.
Uncoated
FDM
FDM + E-4031

Figure 4

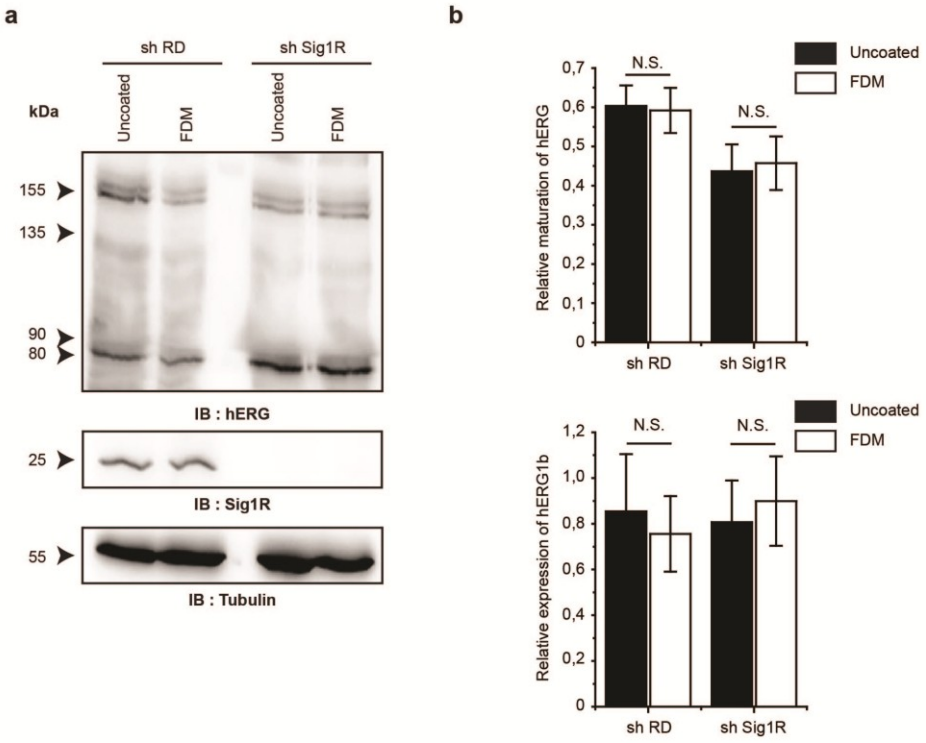

a
sh RD - uncoated
sh RD - FDM
sh RD - FDM + E-4031
sh RD - FDM + LY294002
sh Sig1R - uncoated
sh Sig1R - FDM
sh Sig1R - FDM + E-4031
sh Sig1R - FDM + LY294002
10 μm
b
Traveled length (μm)
Traveled distance (μm)
sh RD
sh Sig1R
N.S.
Uncoated
FDM
FDM + E-4031
FDM + LY294002
c
sh RD
sh Sig1R
Uncoated
FDM
FDM + E-4031
FDM + LY294002
Mean of actin spikes per cell
N.S.


a
2h: discarding of false-positives
48h: Obs. of evading K562
Zf embryos
2 dpf
Injection of K562 in yolk sac
Positive / Negative for invasion
b
Negative
Positive
*
(174)
(81)
% of zebrafish embryos with invasives cells
sh RD
sh Sig1R
c
***
***
Control
E-4031
VEGF secretion (pg / mL / 10^6 cells) x 1000
N.S.
sh RD
sh Sig1R
d
Injection of K562 in the perivitelline space
24h: Obs. of angiogenesis
Zf embryos
2 dpf
Positive / Negative for tumor angiogenesis
e
Negative
Positive
*
(38)
(56)
Negative
Positive
% of embryos
sh RD
sh Sig1R
f
*
% of transmigrated cells
sh RD
sh Sig1R
g
Injection of K562 (i.v. in tail)
24h. Lung harvesting and cell couting
Quantification of lung extravasation
Hairless NOD-SCID mice
30 mn. Control of injection
h
30 mn
24h
sh RD
sh Sig1R
*
Number of extravasating cells per field
sh RD
sh Sig1R

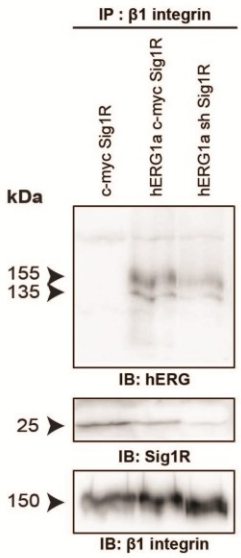
Supplemental Figure 1



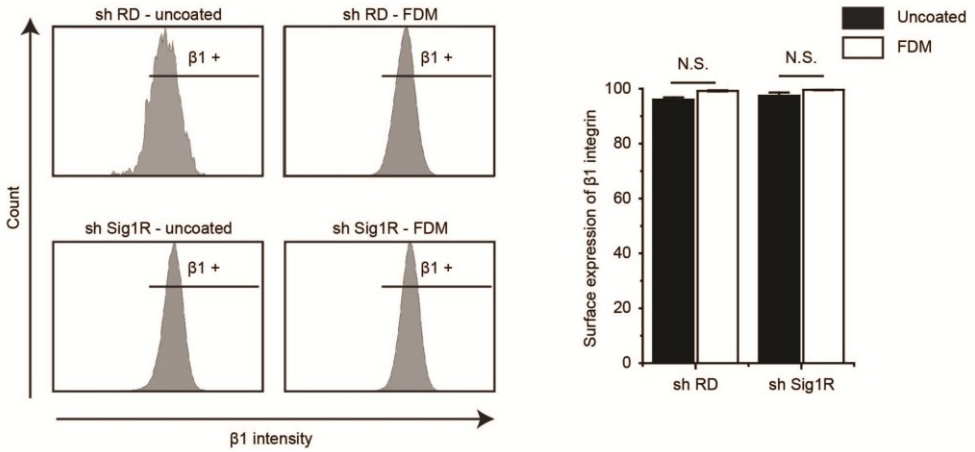
Supplemental Figure 2



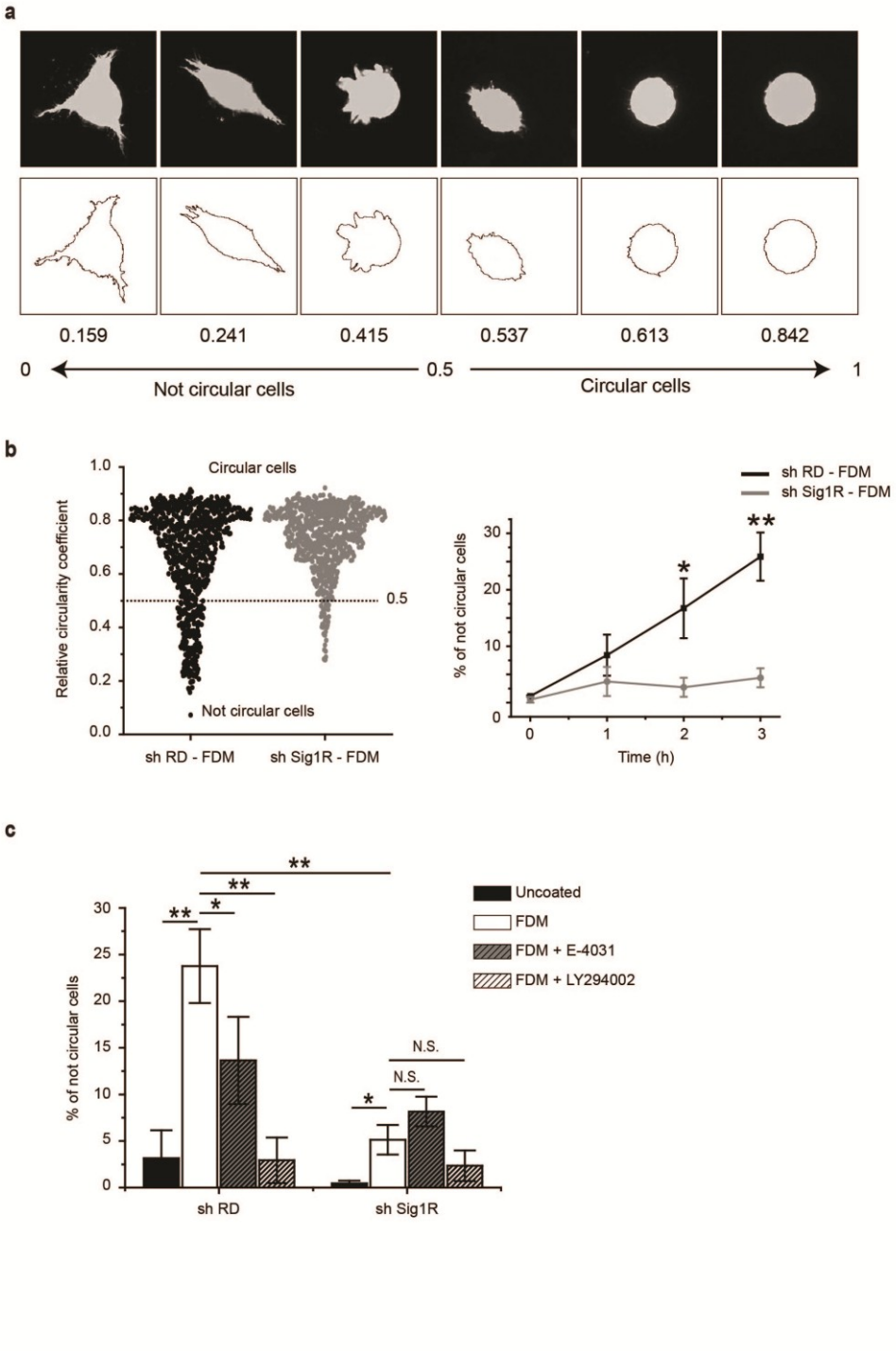
Supplemental Figure 3

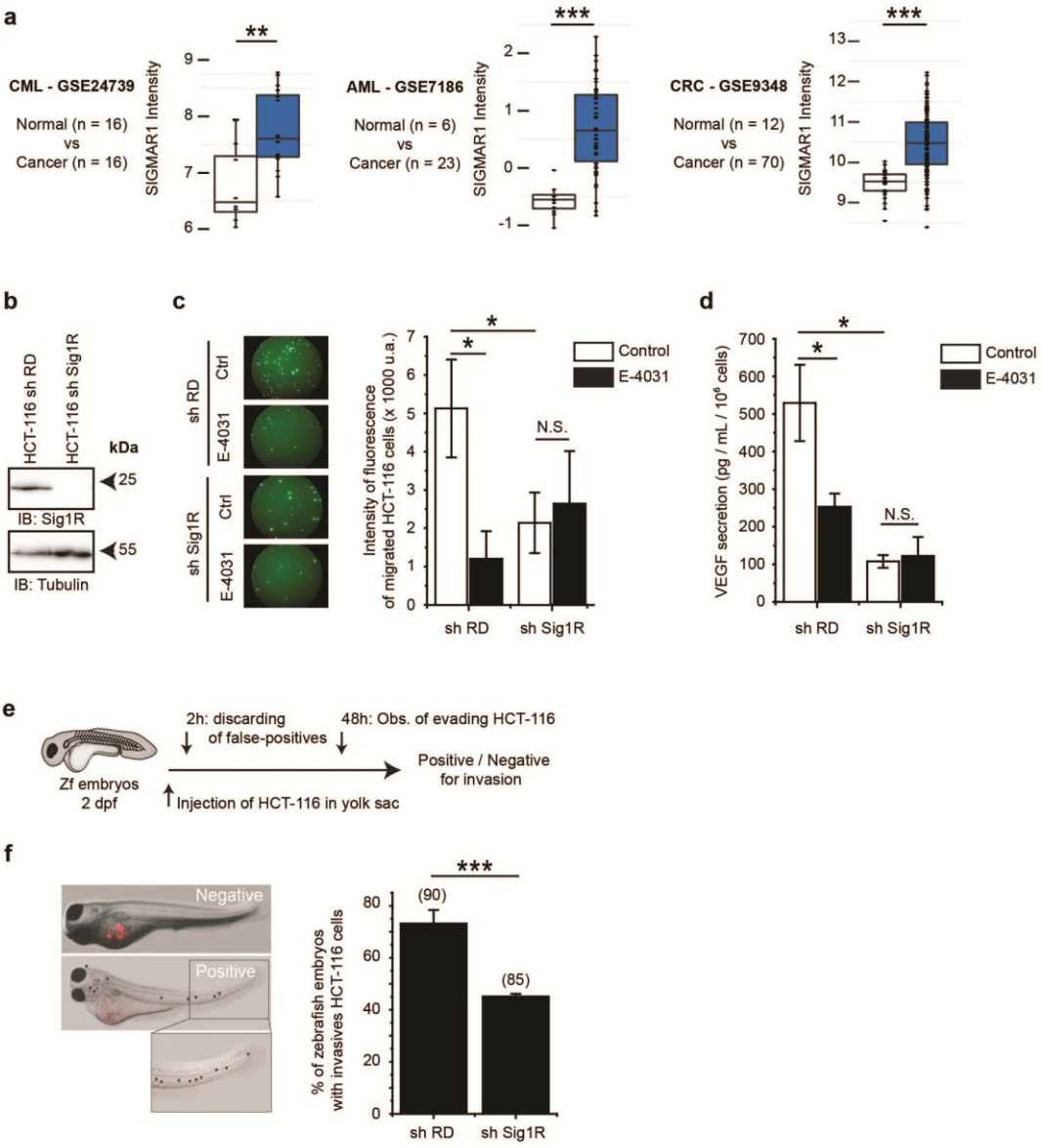


Supplemental Figure 4



Supplemental Figure 5




a
CML - GSE24739
Normal (n = 16)
vs
Cancer (n = 16)
SIGMAR1 Intensity
**
AML - GSE7186
Normal (n = 6)
vs
Cancer (n = 23)
SIGMAR1 Intensity
***
CRC - GSE9348
Normal (n = 12)
vs
Cancer (n = 70)
SIGMAR1 Intensity
***
b
HCT-116 sh RD
HCT-116 sh Sig1R
kDa
25
IB: Sig1R
55
IB: Tubulin
c
sh RD
Ctrl
E-4031
sh Sig1R
Ctrl
E-4031
Intensity of fluorescence of migrated HCT-116 cells (x 1000 u.a.)
*
*
N.S.
Control
E-4031
sh RD
sh Sig1R
d
VEGF secretion (pg / mL / 10^6 cells)
*
*
N.S.
Control
E-4031
sh RD
sh Sig1R
e
Zf embryos
2 dpf
2h: discarding
of false-positives
48h: Obs. of evading HCT-116
Positive / Negative
for invasion
Injection of HCT-116 in yolk sac
f
Negative
Positive
% of zebrafish embryos with invasives HCT-116 cells
***
(90)
(85)
sh RD
sh Sig1R

3. Discussion :

Dans les cellules cancéreuses, le canal hERG est localisé au centre d'une importante plateforme protéique impliquant notamment la sous-unité β1 (β1) des intégrines. L'interaction de la cellule avec les éléments du microenvironnement (fibronectine, VEGF, SDF-1) active hERG qui s'associe aux récepteurs de ces éléments (respectivement β1, Flt-1 et CXCR4). Dans ces macrocomplexes, l'activité de hERG soutient les voies de signalisation sous-jacentes et initie des réponses cellulaires permettant *in fine*, la migration, l'invasion, l'induction de l'angiogenèse tumorale et la dissémination dans l'organisme (Crociani et al., 2013b; Pillozzi et al., 2007; Pillozzi et al., 2011b).

Au cours de la première année d'étude de ma thèse, j'ai mis en évidence le rôle de Sig1R sur l'adressage du canal hERG à la membrane plasmique dans des cellules leucémiques (V.A. De plus, il est démontré que Sig1R module l'activité de la sous-unité β1 des intégrines dans des cellules cancéreuses mammaires (Palmer et al., 2007).

Dans la seconde partie de mon travail sur l'étude de la relation Sig1R / hERG, je me suis donc intéressé au rôle de Sig1R dans l'activité ionique des cellules cancéreuses induites lors de leur interaction avec un élément du microenvironnement tumoral, la matrice extracellulaire (MEC).

J'ai ainsi pu observer que l'interaction des cellules leucémiques avec une matrice extracellulaire (MEC) stimule fortement l'activité ionique de hERG confirmant les observations précédentes (Cherubini et al., 2005). Cette stimulation est dépendante de l'activation de la sous unité β1 des intégrines et de l'expression de Sig1R. Au niveau moléculaire, j'ai pu apprécier que la MEC stimule l'adressage rapide de canaux hERG à la surface de la cellule, renforce l'interaction de hERG avec β1 et initie la voie de signalisation PI3K/Akt. Ce mécanisme est entièrement dépendant de la présence de Sig1R.

Les conséquences de ce mécanisme dépendant de Sig1R sont que l'expression de Sig1R soutient *in vitro* la migration induite par la MEC et stimule la sécrétion du VEGF. *In vivo*, on a constaté que l'inhibition de l'expression de Sig1R réduit le pouvoir invasif, l'angiogenèse induite par les cellules cancéreuses et leur dissémination dans l'organisme.

Ainsi, dans notre étude, Sig1R apparait comme un élément clé de la réponse moléculaire induite par le contact de la cellule avec la MEC. Sig1R augmente la quantité de canaux hERG à la membrane plasmique lors du contact de la cellule avec la MEC. Par ailleurs, Sig1R intervient dans la formation du complexe hERG / β1 et l'initiation de la voie de signalisation PI3K/Akt qui en découle.

Nos travaux identifient donc une réorganisation rapide induite par l'interaction de la cellule avec la MEC de la quantité de canaux hERG exprimés à la surface de la cellule. La membrane de la cellule cancéreuse est donc un système dynamique et plastique. Cette plasticité électrique permet une réponse dynamique et adaptée au microenvironnement.

Par analogie aux travaux de Kourrich et al. qui observent une réorganisation médiée par Sig1R de la quantité de canaux $K_v1.2$ exprimée à la surface des cellules nerveuses lors de la prise répétée de cocaïne (Kourrich et al., 2013; Kourrich et al., 2012). Nous proposons un rôle de Sig1R dans la plasticité électrique des cellules cancéreuses (Crottes et al., 2013) (Annexe 2).

De plus, le rôle observé de Sig1R dans la formation du complexe hERG / β1 tend à prouver que Sig1R joue vraisemblablement un rôle d'adaptateur essentiel dans la formation de complexe protéique impliquant des canaux ioniques.

Pour aller plus loin, il serait intéressant de vérifier ce concept sur les autres partenaires protéiques de hERG. En effet, dans des leucémies myéloïdes et lymphoïdes, hERG peuvent s'associer respectivement avec le récepteur au VEGF, Flt-1, et le récepteur au SDF-1, CXCR4 (Pillozzi et al., 2007; Pillozzi et al., 2011b). Ainsi, il pourrait être intéressant de vérifier dans ces modèles que Sig1R favorise l'association de hERG avec ces récepteurs lorsqu'ils sont stimulés par leurs ligands respectifs.

Un point de notre étude reste cependant à éclaircir. En effet, nos expériences se concentrent uniquement sur le rôle de Sig1R dans l'interaction de hERG avec la sous-unité β1 des intégrines. Or, les récepteurs des intégrines se composent de la dimérisation d'une sous-unité α avec une sous-unité β. Cette hétérodimérisation est essentielle pour que le récepteur soit fonctionnel et exprimé à la surface de la cellule. Il existe plus de 20 combinaisons différentes de sous-unité α et β, chacunnes avec des spécificités différentes pour des ligands présents dans le microenvironnement (tels que la fibronectine, la vitronectine, la laminine, etc…) ou d'autres récepteurs cellulaires (ICAM-1, VCAM-1, etc ...). Cette diversité des intégrines leur permet d'initier des réponses différentes et donc permet de moduler des fonctions physiologiques différentes (Cox et al., 2010; Huttenlocher and Horwitz, 2011; Margadant et al., 2011).

Notre modèle de lignée cellulaire, K562, est décrit pour exprimer uniquement les intégrines α5β1 (Delwel et al., 1993). Nos expériences indiquent que Sig1R n'influe pas sur l'expression à la surface de la sous-unité β1 des intégrines et donc que Sig1R n'intervient pas dans l'association de la sous-unité α5 avec la sous-unité β1 et de l'expression à la

surface de cet hétérodimère α5β1. Ainsi, Sig1R exercerait son rôle d'adaptateur uniquement sur l'interaction de hERG avec la sous-unité β1 de l'hétérodimère α5β1.

Cependant, dans ces cellules qui n'expriment que l'hétérodimère α5β1, il a été montré qu'un traitement chronique de cette lignée cellulaire avec un agent chimiothérapeutique, l'Imatinib (inhibiteur des récepteurs tyrosine kinase) induit l'expression de l'hétérodimère $\alpha_V\beta3$ qui provoque un changement morphologique des cellules et une résistance à la chimiothérapie accrue (Puissant et al., 2012). Cela suggère que l'expression des intégrines est dynamique et peut être modulée de différentes manières. Ainsi, il serait intéressant de regarder dans différentes conditions (traitement chimiothérapeutique, culture continue sur la MEC, etc …) si l'expression de Sig1R peut provoquer de tels changements dynamiques de l'expression à la surface des intégrines et si cela entraine des conséquences sur l'activité électrique de la cellule.

Par ailleurs, dans des modèles cellulaires exprimant plusieurs sous-unités α, la sous-unité β1 des intégrines et le canal hERG, il serait intéressant d'observer si le rôle de Sig1R sur la formation du complexe hERG / β1 est dépendant de la sous-unité α associée.

Les interactions cellules / MEC sont connues pour être cruciales dans la tumorigenèse mais aussi dans le développement tissulaire (Hynes, 2009; Lu et al., 2011; Muschler and Streuli, 2010). Ici, notre étude démontre la dépendance de Sig1R vis-à-vis d'un stimulus extracellulaire, qu'est la MEC. Ainsi, l'expression de Sig1R favorise la réactivité de la cellule dans la mise en place de la plasticité électrique lors de son interaction avec la MEC.

Ceci suggère que Sig1R peut être activé physiologiquement par des stimuli autres que des stress cellulaires et peut par conséquent étendre le spectre des fonctions physiologiques et pathologiques possibles de Sig1R.

Il est intéressant de remarquer que la MEC induit une augmentation de l'activité et de l'expression à la membrane plasmique des canaux hERG sans affecter le niveau d'expression ou de maturation du canal. Sig1R conserve cependant son effet sur le niveau de maturation du canal hERG indépendamment de la présence de la MEC. Ainsi, deux hypothèses peuvent expliquer ces observations.

Dans la première, la MEC stimulerait directement Sig1R, ce qui induirait la mobilisation rapide de canaux hERG à la membrane plasmique, indépendamment de leur état de maturation. Cette hypothèse suggère donc un nouveau mécanisme d'action de Sig1R dans lequel la protéine favorise l'adressage rapide à la membrane plasmique de

canaux ioniques – indépendamment de son effet sur la maturation du canal – en réponse à un stimulus extracellulaire.

L'hypothèse alternative serait un rôle de Sig1R sur la maturation et la stabilité de hERG – que l'on a démontré dans la première partie de notre étude – permettant de créer un « pool » de canaux hERG matures prêts à être acheminés à la membrane plasmique lors de la stimulation par la MEC. La MEC aurait donc un effet supérieur dans les cellules exprimant Sig1R en raison d'un « pool » plus important de canaux hERG mature rapidement disponibles. Le rôle de Sig1R et de la MEC sur hERG seraient alors la manifestation de deux mécanismes indépendants mais complémentaires. Dans cette seconde hypothèse, la MEC n'aurait aucun rôle stimulateur sur Sig1R.

En absence de MEC, nous n'observons pas de différences induites par l'inhibition de l'expression de Sig1R sur l'activité de hERG ou sur la quantité de canaux hERG à la membrane plasmique. Dans ces mêmes conditions, le niveau de maturation du canal hERG est cependant affecté par l'expression de Sig1R. Ceci suggère qu'en absence de MEC, le rôle de Sig1R sur la maturation de hERG ne permet pas de moduler son activité ou son adressage à la membrane plasmique. Ainsi, la MEC stimule l'adressage des canaux hERG à la membrane plasmique de façon indépendante du processus de maturation de hERG. Cela sous-tend l'hypothèse d'un rôle de Sig1R et de la MEC indépendant mais complémentaire.

Cependant, l'augmentation de l'expression de Sig1R à la membrane plasmique et sa colocalisation avec la sous-unité β1 des intégrines induites lors de l'interaction avec la MEC, indiquent que celle-ci peut agir sur Sig1R, renforçant ainsi notre première hypothèse.

On peut aussi proposer la coexistence des mécanismes issus de nos deux hypothèses pour en former une troisième : Sig1R agirait sur la maturation du canal hERG mais ne permettrait pas son expression à la surface en absence de la MEC. L'interaction avec la MEC pourrait ainsi déclencher la mobilisation de Sig1R et des canaux hERG associés à la membrane plasmique. Une fois à la membrane plasmique, Sig1R permettrait la formation du macrocomplexe hERG / β1 (Figure 9).

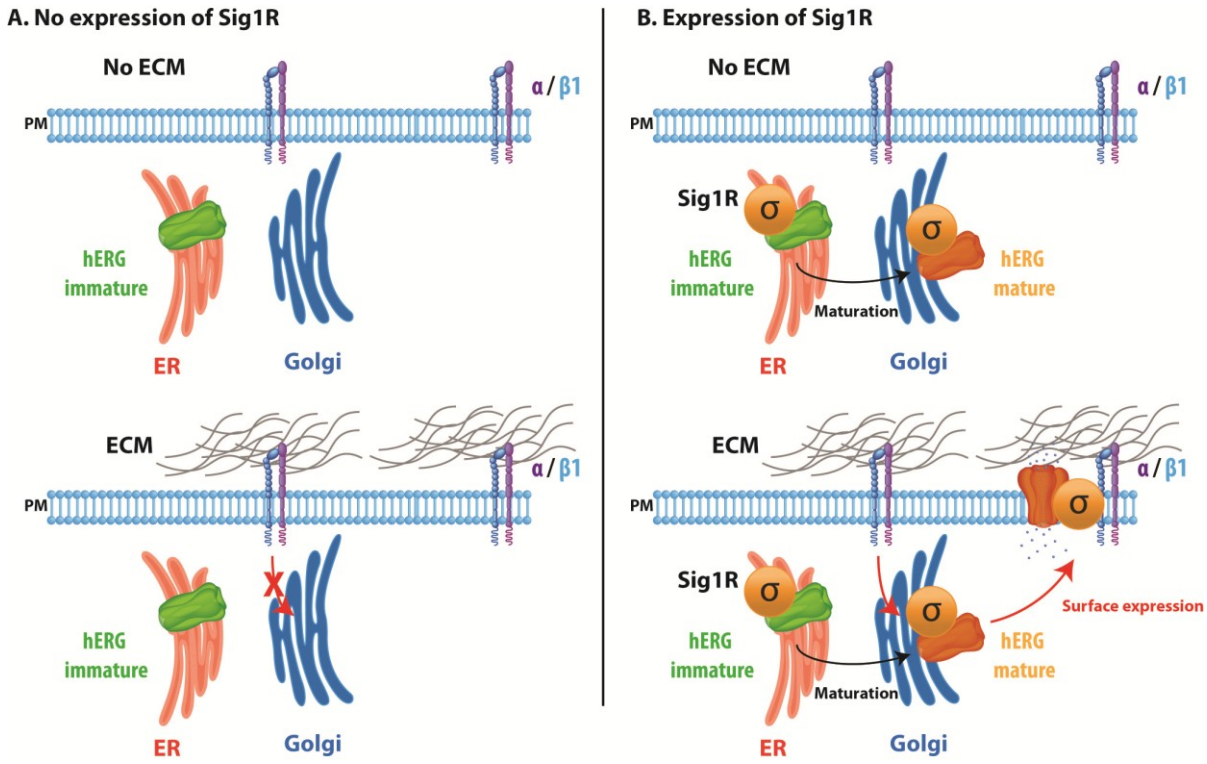


Figure 9 : Représentation schématique du rôle de l'expression de Sig1R dans la plasticité électrique induite par la matrice extracellulaire. (PM : membrane plasmique, ER : réticulum endoplasmique, ECM, matrice extracellulaire, α / β1 : intégrines α_x/β1).

Ainsi, dans cette étude, on observe que la MEC stimule l'adressage de hERG et de Sig1R à la membrane plasmique sans agir sur le niveau de maturation de hERG. En absence de la MEC, le rôle de Sig1R sur la maturation de hERG ne permet pas de favoriser son adressage à la surface.

Toutefois, cette observation est en contradiction avec les résultats de la première étude (V.A.2). En effet, dans cette étude, j'observe dans le même modèle cellulaire que l'expression de Sig1R module l'activité de hERG en facilitant son expression à la surface et sa maturation sans avoir recours à la MEC. Comment peut-on alors expliquer ces deux observations différentes ?

Une réponse à cette question peut se trouver dans les conditions d'études. En effet, dans la première étude, les cellules sont cultivées dans du milieu supplémenté à 5% de sérum de veau fœtal. Dans la seconde étude, les cellules sont cultivées dans du milieu sans sérum. Ainsi, on peut imaginer que le sérum, contenant des facteurs solubles tels que la fibronectine soluble et de nombreux facteurs de croissance, permettent de « stimuler » les cellules et donc de favoriser l'adressage des canaux hERG à la surface à travers l'activation de Sig1R, comme le ferait la MEC.

Au final, notre troisième hypothèse (Figure 9) permet d'expliquer toutes les particularités de nos observations et propose trois rôles différents de Sig1R sur le canal hERG. L'un sur la maturation, le second sur la stabilité et le dernier sur l'adressage à la membrane en réponse à la stimulation par la MEC. Ces trois rôles de Sig1R sur hERG peuvent s'expliquer par l'hypothèse formulée plus haut du rôle « d'adaptateur universel » de Sig1R. Selon les protéines s'associant au complexe Sig1R / hERG, on pourrait observer un rôle différent de Sig1R sur hERG. Des expériences complémentaires seront cependant nécessaires afin de mettre en lumière ce rôle adaptateur de Sig1R sur les canaux ioniques.

Cette étude met également en avant le pouvoir pro-tumoral de Sig1R et le lien avec sa fonction régulatrice des canaux ioniques. En effet, nous observons que l'expression de Sig1R, à travers son action sur l'activité de hERG et l'activation de la voie de signalisation PI3K/AKT qui en découle, favorise le potentiel invasif des cellules leucémiques *in vitro* et *in vivo* en agissant sur les processus de migration, d'invasion, d'angiogénèse et d'extravasation. Cette observation est non seulement valable dans le cadre de leucémie myéloïde chronique mais aussi pour des cellules issues de cancers colorectaux exprimant le canal hERG.

Ces résultats nous permettent donc de démontrer pour la première fois, le rôle de l'expression de Sig1R dans la carcinogenèse et soutiennent notre proposition du ciblage thérapeutique de Sig1R dans les cancers.

Des études sont actuellement en cours afin de valider un rôle inhibiteur des ligands sigma sur l'invasivité des cellules cancéreuses dans nos modèles de xénotransplantation dans l'embryon de poisson-zèbres. Ces études devraient donc permettre de renforcer l'importance de Sig1R dans les cancers mais aussi de poser les bases de futures stratégies thérapeutiques.

C. Sig1R interagit avec le canal $Na_v1.5$ et module son activité dans des cellules cancéreuses mammaires

1. Introduction :

Le canal $Na_v1.5$ est un canal sodique voltage-dépendant qui intervient notamment dans l'initiation du potentiel d'action. Comme pour hERG, différentes mutations de ce canal ont été observées chez des patients souffrant de maladies cardiaques telles que le syndrome de QT long ou le syndrome de Brugada (Moreau et al., 2014; Rook et al., 2012; Yu et al., 2005).

A la différence du canal hERG, qui est un tétramère, la sous-unité α du canal $Na_v1.5$ est un monomère avec quatre domaines composés chacun de six segments transmembranaires et d'une boucle P entre les segments 5 et 6. L'arrangement tridimensionnel de ces quatre domaines permet de réunir les quatre boucles P et de créer un pore ionique sélectif au sodium. La sous-unité α du canal peut s'associer à différentes sous-unités auxiliaires β qui peuvent influer sur les caractéristiques biophysiques.

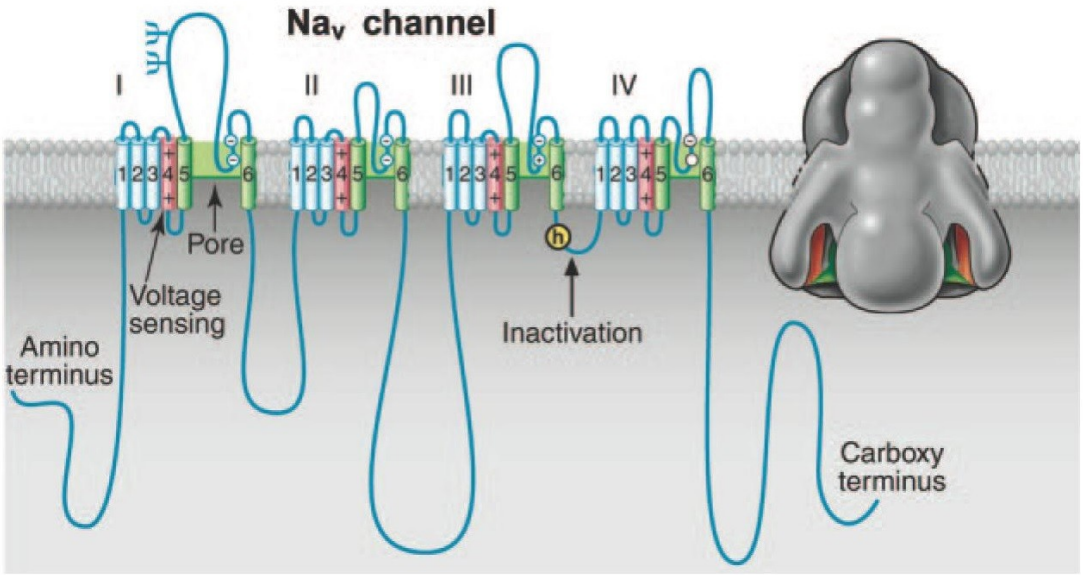


Figure 8 : Structure de la sous-unité α du canal sodique voltage-dépendant $Na_v1.5$ (D'après Yu et al., 2005). Gauche : représentation schématique de la structure de la sous-unité α du canal $Na_v1.5$. Les cylindres numérotés représentent les hélices transmembranaires. Les chiffres romains indiquent les quatre domaines transmembranaires du canal. Droite : Structure 3D de la sous-unité α du canal $Na_v1.5$ à une résolution de 20 Å (d'après Sato et al. 2001 et Yu et Catterall 2004).

$Na_v1.5$ est surexprimé dans des neuroblastomes, des cancers mammaires, pulmonaires, colorectaux et ovariens (Brackenbury, 2012). Son expression est corrélée à l'agressivité de la tumeur (Fraser et al., 2005; Gao et al., 2010) et participe au potentiel invasif des cellules (Brackenbury, 2012; Brisson et al., 2011; Gillet et al., 2009; Roger et al., 2003).

L'invasion cellulaire est la résultante de deux fonctions : la migration et la dégradation du milieu extracellulaire.

Le rôle du canal $Na_v1.5$ dans la migration cellulaire est discuté. En effet, l'inhibition du canal par la tétrodotoxine (TTX) dans la lignée cellulaire dérivée de cancer du sein, MDA-MB-231 n'induit aucun effet sur la migration des cellules, mais inhibe fortement leur capacité à dégrader la matrice extracellulaire (Roger et al., 2003). Dans une autre étude, l'inhibition du canal $Na_v1.5$ par la TTX réduit la migration de la même lignée cellulaire (Fraser et al., 2005). Une étude récente démontre que le canal $Na_v1.5$ participe à la migration cellulaire dans ces mêmes cellules (Yang et al., 2012a). De même, de nouvelles données indiquent que le canal $Na_v1.5$ intervient dans la polymérisation de l'actine et la morphologie cellulaire (Brisson et al., 2013). Ces éléments suggèrent que le canal $Na_v1.5$ pourrait participer à la migration cellulaire, en permettant les changements morphologiques nécessaires au mouvement de la cellule. Enfin, une étude combinant analyses génomiques et bioinformatiques met en évidence le rôle potentiel de ce canal dans la migration des cellules cancéreuses du colon, à travers une action sur la voie de signalisation Wnt (House CD. 2010). Pour le moment, aucune confirmation de l'intervention de $Na_v1.5$ sur cette voie de signalisation n'a été publiée.

A contrario, le rôle de $Na_v1.5$ dans la dégradation de la matrice extracellulaire par la cellule cancéreuse est unanimement reconnu (Brisson et al., 2013; Brisson et al., 2011; Fraser et al., 2005; Gillet et al., 2009; Roger et al., 2003). Localisé dans les invadopodes (petites excroissances de la cellule spécialisées dans la digestion de la matrice extracellulaire), le canal $Na_v1.5$ y est associé à l'échangeur ionique sodium/proton, NHE1, mais aussi avec la cavéoline-1, marqueur des radeaux lipidiques. Dans ce macrocomplexe, l'activité de $Na_v1.5$ permet de favoriser l'efflux de protons (ions H^+) généré par NHE1. Cet efflux de protons va créer une acidification locale du milieu environnant et conduire à l'activation des cathepsines B qui vont alors digérer la matrice extracellulaire (Brisson et al., 2013; Brisson et al., 2011; Gillet et al., 2009).

En plus de son rôle dans l'invasion, le canal $Na_v1.5$ favorise l'activation de la kinase Src ainsi que de la cortactine, deux acteurs très impliqués dans l'agressivité des cellules cancéreuses (Brisson et al., 2013)(Brisson 2013). La GTPase RhoA, dont l'expression est corrélée à l'agressivité tumorale, semble également avoir un lien étroit avec le canal $Na_v1.5$ en agissant sur son expression (Dulong et al., 2014).

Ainsi, le canal $Na_v1.5$ participe à la progression métastatique des cellules cancéreuses en agissant principalement sur les mécanismes permettant l'invasion cellulaire, favorisant la migration cellulaire et la dégradation de la matrice extracellulaire aux extrémités des invadopodes.

Dans une série de travaux récents, l'équipe de Ruoho a montré l'existence d'un lien fonctionnel entre Sig1R et le canal $Na_v1.5$ dans le tissu cardiaque (Fontanilla et al., 2009; Johannessen et al., 2011; Johannessen et al., 2009) (III.E.2). Par ailleurs, une série d'études réalisées par l'équipe du Dr. Mike Edwardson (Cambridge, Royaume-Uni) a permis d'identifier l'interaction directe de Sig1R avec certains canaux ioniques (récepteur au NMDA, canal ASIC1a). Dans cette étude réalisée en collaboration avec le Dr. Mike Edwardson, nous avons cherché à addressé la question de l'interaction de Sig1R avec le canal Nav1.5 et les conséquences de cette interaction sur les courants sodiques des cellules issues de cancers du sein.

2. Article : The Sigma-1 Receptor Binds to the Nav1.5 Voltage-gated Na^+ Channel with 4-Fold Symmetry

⌘ *Author's Choice*

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The Sigma-1 Receptor Binds to the Nav1.5 Voltage-gated Na^+ Channel with 4-Fold Symmetry*

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Background: The sigma-1 receptor modulates the activity of ion channels.
Results: Atomic force microscopy imaging of complexes between sigma-1 receptors and Nav1.5 Na^+ channels reveals a 4-fold symmetry.
Conclusion: Each of the four sets of six transmembrane regions in Nav1.5 constitutes a sigma-1 receptor binding site.
Significance: The sigma-1 receptor likely interacts with the transmembrane regions of its protein partners.

The sigma-1 receptor (Sig1R) is up-regulated in many human tumors and plays a role in the control of cancer cell proliferation and invasiveness. At the molecular level, the Sig1R modulates the activity of various ion channels, apparently through a direct interaction. We have previously shown using atomic force microscopy imaging that the Sig1R binds to the trimeric acid-sensing ion channel 1A with 3-fold symmetry. Here, we investigated the interaction between the Sig1R and the Nav1.5 voltage-gated Na^+ channel, which has also been implicated in promoting the invasiveness of cancer cells. We show that the Sig1R and Nav1.5 can be co-isolated from co-transfected cells, consistent with an intimate association between the two proteins. Atomic force microscopy imaging of the co-isolated proteins revealed complexes in which Nav1.5 was decorated by Sig1Rs. Frequency distributions of angles between pairs of bound Sig1Rs had two peaks, at ~90° and ~180°, and the 90° peak was about twice the size of the 180° peak. These results demonstrate that the Sig1R binds to Nav1.5 with 4-fold symmetry. Hence, each set of six transmembrane regions in Nav1.5 likely constitutes a Sig1R binding site, suggesting that the Sig1R interacts with the transmembrane regions of its partners. Interestingly, two known Sig1R ligands, haloperidol and (+)-pentazocine, disrupted the Nav1.5/Sig1R interaction both *in vitro* and in living cells. Finally, we show that endogenously expressed Sig1R and Nav1.5 also functionally interact.

The sigma-1 receptor (Sig1R)[3] is widely expressed in both the central nervous system and peripheral tissues (1, 2), and a variety of functions have been ascribed to it, including modulation of voltage-gated (3–11), ligand-gated (12–15), volume-regulated (16) and acid-sensitive (17) ion channel activity at the plasma membrane, control of Ca^{2+} release from the endoplasmic reticulum (18), and neuroprotection in cerebral ischemia and stroke (19). Of most relevance to the present study, the Sig1R is known to be up-regulated in many human tumors and has been implicated in the control of cancer cell proliferation, resistance to apoptosis, and invasiveness (16, 20, 21).

The Sig1R shares 30% identity and 67% similarity with a yeast sterol C8-C7 isomerase (ERG2; 22). The receptor contains two transmembrane regions, although it is still unclear whether the N and C termini are cytoplasmic (5) or extracytoplasmic (18). The Sig1R is modulated by a wide variety of ligands (1, 2), including antipsychotic drugs (*e.g.* haloperidol) and psychotomimetics (*e.g.* pentazocine). Indeed, most of the evidence for the physiological relevance of the Sig1R has relied on the effects of these ligands; in contrast, there have been relatively few reports of functional effects of ligand-free Sig1Rs (*e.g.* Refs. 5, 11, 16, 18). Evidence has been presented recently that the hallucinogen *N*,*N*-dimethyltryptamine is an endogenous ligand at the Sig1R (23).

There is some evidence that modulation of ion channel function by the Sig1R is direct; for instance, its effects on the Kv1.4 voltage-gated K^+ channel do not involve transduction mechanisms such as G protein signaling or phosphorylation (24). Furthermore, the Sig1R can be co-immunoprecipitated with Kv1.4 from membrane lysates prepared from both rat posterior pituitary cells and mRNA-injected *Xenopus* oocytes (5), and with the human ether-à-go-go-related gene (hERG) potassium channel from hERG/Sig1R-transfected human embryonic kidney (HEK)-293 cells (11). Using atomic force microscopy (AFM) imaging, we recently demonstrated at the single mole-

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[3] The abbreviations used are: Sig1R, sigma-1 receptor; AFM, atomic force microscopy; ASIC, acid-sensing ion channel; hERG, human ether-à-go-go-related gene; TMR, transmembrane region.

cule level that the Sig1R directly interacts with the acid-sensing ion channel (ASIC)1a, which is known to assemble as a trimer (25, 26), forming a complex with 3-fold symmetry (27). This interaction likely underlies the ability of the Sig1R endogenously expressed in rat cortical neurons to inhibit ASIC1a-mediated membrane currents and thereby reduce consequent intracellular Ca^{2+} accumulation (17).

In the present study, we focused on the interaction between the Sig1R and the voltage-gated Na^+ channel Nav1.5, which incorporates four sets of six transmembrane regions (TMRs) within a single polypeptide (28). Nav1.5 is responsible for the rapid upstroke of the action potential in cardiac myocytes and for the rapid propagation of the cardiac action potential. Significantly, it is also implicated in promoting the invasiveness of breast cancer cell lines such as MDA-MB-231 (29–31) and is known to be modulated by Sig1R ligands (3, 4, 23). For example, (+)-pentazocine reversibly inhibited Nav1.5 channels stably expressed in HEK-293 cells and in cardiac myocytes but had a much smaller effect in Sig1R knock-out myocytes (3). *N*,*N*-Dimethyltryptamine, the putative endogenous Sig1R ligand, had similar inhibitory effects on Nav1.5 currents and induced hypermobility in mice that was abrogated when the Sig1R was knocked out (23). In contrast, progesterone acted as an antagonist at the Sig1R, blocking the inhibitory effects of ligands such as *N*,*N*-dimethyltryptamine on Nav1.5 (4).

We set out to examine the structure of the complex formed between the Sig1R and Nav1.5 and to test whether the Sig1R/Nav1.5 interaction has functional consequences for Nav1.5 activity in MDA-MB-231 breast cancer cells. We show using AFM imaging that the Sig1R binds to Nav1.5 with 4-fold symmetry, suggesting that the Sig1R interacts with its partner proteins via their TMRs. We also show that Nav1.5 currents in MDA-MB-231 cells fall when Sig1R expression is reduced.

EXPERIMENTAL PROCEDURES

Cell Culture—tsA 201 cells (a subclone of HEK-293 cells stably expressing the SV40 large T-antigen) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in an atmosphere of 5% CO_2 and 95% air. The breast cancer cell line, MDA-MB-231, kindly provided by Dr. Laurent Combettes (Université de Paris-Sud), was cultured in DMEM supplemented with 10% fetal bovine serum and 50 units/ml penicillin and 50 μg/ml streptomycin.

Constructs—cDNA encoding the human Sig1R, with a C-terminal FLAG epitope tag, was subcloned into the vector pcDNA3.1/V5-His using HindIII and AgeI so as to delete the V5 epitope tag but leave the His_6 tag. (The His_6 tag was not used in any of the experiments described here.) The sequence of the construct was verified before use. cDNA encoding human Nav1.5, with a C-terminal hemagglutinin (HA) epitope tag, in the vector pcDNA3N, was kindly provided by Dr. C. Valdivia (University of Michigan).

Transient Transfection of tsA 201 Cells—Transient transfections of tsA 201 cells with DNA were carried out using the calcium phosphate precipitation method. A total of 250 μg of DNA was used to transfect cells in 5 × 162 cm^2 culture flasks. For co-transfections with Sig1R-FLAG and Nav1.5-HA, 125 μg of DNA for each construct was used. After transfection, cells were incubated for 48 h at 37 °C to allow protein expression. Protein expression and intracellular localization were checked using immunofluorescence analysis of small-scale cultures. Cells were fixed, permeabilized, and incubated with appropriate primary antibodies (mouse monoclonal anti-FLAG (Sigma), mouse monoclonal anti-HA (Covance), rabbit polyclonal anti-HA (Sigma), and mouse monoclonal anti-Myc (Invitrogen), as a negative control), followed by either Cy3-conjugated goat anti-mouse, Cy3-conjugated goat anti-rabbit or fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies (Sigma). Cells were imaged by confocal laser scanning microscopy. To test for endogenous expression of the Sig1R, cell extracts were immunoblotted using a rabbit anti-Sig1R polyclonal antibody (Abcam). Immunoreactive bands were visualized using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody followed by enhanced chemiluminescence.

Solubilization and Purification of Epitope-tagged Proteins—Transfected cells were solubilized in 1% Triton X-100 for 1 h, before centrifugation at 61,740 × *g* to remove insoluble material. To capture Nav1.5-HA, the solubilized extract was incubated with anti-HA-agarose beads (Sigma) for 3 h. The beads were washed extensively, and bound proteins were eluted with HA peptide (100 μg/ml). Sig1R-FLAG was captured in the same way using anti-FLAG-agarose beads and a triple-FLAG peptide (Sigma). Samples were analyzed by SDS-PAGE, followed by Coomassie blue staining and/or immunoblotting, using mouse monoclonal antibodies against HA or FLAG. Where appropriate, band densitometry was carried out using NIH ImageJ software (http://rsbweb.nih.gov/ij/).

Isolation of Biotinylated Proteins—To establish whether Sig1R-FLAG and Nav1.5-HA interacted at the plasma membrane, intact co-transfected cells were biotinylated by incubation with sulfo-NHS-LC-biotin (Pierce; 1 mg/ml) for 30 min. A detergent extract of the cells was then produced as described above. Biotinylated proteins were incubated with monomeric avidin-agarose (Pierce) for 1 h. The beads were washed extensively, and bound proteins were eluted with free biotin (2 mM). Eluted Sig1R-FLAG was captured using anti-FLAG-agarose beads and eluted with triple-FLAG peptide (Sigma). Samples were analyzed by SDS-PAGE, followed by immunoblotting, using mouse monoclonal antibodies against HA and FLAG.

AFM Imaging of Isolated Proteins—Isolated protein samples were diluted to a final concentration of 0.04 nM, and 45 μl of the sample was allowed to adsorb to freshly cleaved mica disks. After a 5-min incubation, the sample was washed with Biotechnology Performance Certified-grade water (Sigma) and dried under nitrogen. Imaging was performed with a Veeco Digital Instruments Multimode AFM controlled by a Nanoscope IIIa controller. Samples were imaged in air, using the tapping mode. The silicon cantilevers used had a drive frequency ~300 kHz and a specified spring constant of 40 N/m (Olympus). The applied imaging force was kept as low as possible (A_s/A_0 ~0.85).

For individual Sig1R particles, molecular volumes were determined using Scanning Probe Image Processor (version 5; Image Metrology). It is well known that the geometry of the scanning AFM probe introduces a tendency to overestimate

particle diameter. To minimize this probe convolution error, we used a Scanning Probe Image Processor particle threshold of 0.1 nm to provide accurate measurements of diameter. For particles within complexes, particle heights and diameters were measured manually using the Nanoscope software and used to calculate molecular volumes, according to Equation 1,

$$V_m = (\pi h/6)(3r^2 + h^2) \quad \text{(Eq. 1)}$$

where h is the particle height and r is the radius (32). This equation assumes that the adsorbed particles adopt the form of a spherical cap.

Molecular volume based on molecular mass was calculated using Equation 2,

$$V_c = (M_0/N_0)(V_1 + dV_2) \quad \text{(Eq. 2)}$$

where M_0 is the molecular mass, N_0 is Avogadro's number, V_1 and V_2 are the partial specific volumes of particle (0.74 cm^3/g) and water (1 cm^3/g), respectively, and d is the extent of protein hydration (taken as 0.4 g water/g protein).

Selection of Binding Events—Several criteria were used to identify Nav1.5·Sig1R complexes. Heights and radii were measured for all particles, and the particle volumes were calculated. Bound particles needed to have a molecular volume between 30 and 120 nm^3, which was the experimentally determined volume range for a Sig1R. A cross-section was drawn through the junction between the Sig1R and the adjacent Nav1.5 channel, and the height of the lowest point between receptor and channel was measured. This height needed to be greater than 0.3 nm for the Sig1R to be considered bound. Any particle was rejected if its length was greater than twice its width. To be considered a double binding event, all particles and both binding events needed to meet all of the above criteria.

Note that it has been shown previously (32) that the molecular volumes of proteins measured by imaging in air are similar to the values obtained by imaging under fluid; hence, the process of drying does not significantly affect the measured molecular volume. It has also been shown by the authors (33) and by others (32) that there is a close correspondence between the measured and predicted molecular volumes for various proteins over a wide range of molecular masses; hence, molecular volume is measured reasonably accurately by AFM imaging.

Statistical Analysis—Histograms were drawn with bin widths chosen according to Scott's equation,

$$\text{Bin width} = 3.5\sigma/n^{1/3} \quad \text{(Eq. 3)}$$

where σ is an estimate of the S.D. and n is the sample size (34). Where Gaussian curves were fitted to the data, the number of curves was chosen so as to maximize the r^2 value while giving significantly different means using Welch's t test for unequal sample sizes and unequal variances (35).

In Situ Proximity Ligation Assay—Cells growing on lysine- and collagen-coated glass coverslips in 3.5-cm diameter culture wells were co-transfected with 1.5 μg each of DNA encoding Nav1.5-HA and Sig1R-FLAG. Cells were incubated for 24 h at 37 °C to allow protein expression. Cells were fixed, permeabilized, and incubated with primary antibodies (rabbit polyclonal anti-HA plus mouse monoclonal anti-FLAG, both diluted 1:50) for a further 1 h. Cells were washed with phosphate-buffered saline and then incubated for 1 h with anti-mouse (+) and anti-rabbit (−) proximity ligation secondary antibodies, diluted 1:5, at 37 °C (36). These antibodies were obtained as part of a kit from Olink Bioscience, which also included ligation and amplification buffers. Cells were washed with phosphate-buffered saline and incubated in T4 DNA ligase diluted 1:40 in ligation buffer for 30 min at 37 °C. Cells were washed with TBS-T (150 mM NaCl, 0.05% Tween 20, 10 mM Tris, pH 7.4), and DNA amplification was performed by incubation with 1:80 DNA polymerase in amplification buffer for 100 min at 37 °C. This buffer also contained a fluorescent detection probe (excitation wavelength, 554 nm; emission wavelength, 576 nm). Cells were washed with 1× TBS (100 mM NaCl, 20 mM Tris, pH 7.5) and then 0.01× TBS. Coverslips were mounted on slides and imaged by confocal laser scanning microscopy.

Small Hairpin (sh)RNA Transduction of MDA-MB-231 Cells—The shRNA sequences targeted either to the Sig1R (GACTTCCTCACCCTCTTCTATCTCGAGATAGAAGAGGGTGAGGAAGTC) or to a random sequence (CAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGT) were subcloned into the mammalian expression vector pPRIpu as described previously (37) to generate the constructs pPRIGpu-shSig1R and pPRIGpu-shRD, respectively. Highly purified recombinant plasmids were obtained by anion-exchange chromatography (NucleobondAx, Macherey-Nagel). To generate retroviruses, HEK-293T cells were transfected the following day with 10 μg of either pPRIGpu-shSig1R or pPRIGpu-shRD, together with 5 μg of plasmid pCMV-VSV G and 5 μg of plasmid pCMV-gag-pol, using the calcium phosphate precipitation method. Six hours after transfection, cells were washed, and fresh medium was added. Replication-defective retroviruses were recovered in the culture medium between 24 h and 72 h post-transfection. For transduction experiments, MDA-MB-231 cells were seeded at 30–40% density in 100-mm dishes in culture medium. Retroviral supernatants were filtered through sterile 0.45-μm filters and then added directly to MDA-MB-231 cells in the presence of 4 μg/ml hexadimethrin bromide to enhance retroviral transduction efficiency. On day 6, puromycin (4 μg/ml) was added in fresh medium to begin selection of transduced cells.

Electrophysiology Experiments—MDA-MB-231 cells were prepared as previously described (29). The physiological saline solution used contained 5 mM KCl, 140 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES, pH 7.4. The pipette solution contained 130 mM potassium aspartate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 2 mM ATP, 10 mM HEPES, pH 7.2. Soft glass patch electrodes (Brand, Wertheim, Germany) were prepared on a horizontal pipette puller (P-97, Sutter Instrument Co., Novato, CA) to achieve a final resistance of 2.5–4.0 megohms. Currents were recorded in whole cell configuration and voltage clamp mode at room temperature using an Axopatch 200B patch clamp amplifier (Axon instruments) with a DIGIDATA 1440 interface and pClamp10.2 software (Axon instruments). Analog signals were sampled at 10 kHz and filtered at 2 kHz. Cell capacitance and series resistance were electronically compensated by ~60%. The P/5 subpulse correction of cell leakage was used to study the Na^+ current. Na^+ current-

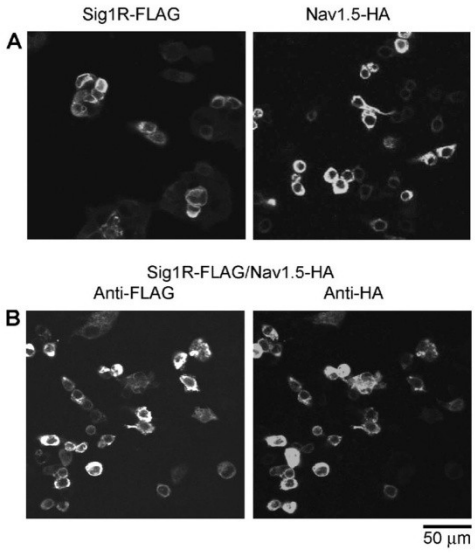

FIGURE 1. **Expression of the Sig1R and Nav1.5 in transfected tsA 201 cells.** *A*, cells were singly transfected with either Sig1R-FLAG (*left panel*) or Nav1.5-HA (*right panel*). Cells were fixed, permeabilized and incubated with either mouse monoclonal anti-FLAG or mouse monoclonal anti-HA antibodies, followed by a Cy3-conjugated goat anti-mouse secondary antibody. Cells were imaged by confocal laser scanning microscopy. *B*, cells were co-transfected with Sig1R-FLAG and Nav1.5-HA. Cells were fixed, permeabilized, and incubated with both mouse monoclonal anti-FLAG and rabbit polyclonal anti-HA antibodies, followed by fluorescein isothiocyanate-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit secondary antibodies. Cells were imaged by confocal laser scanning microscopy. *Scale bar*, 50 μm.

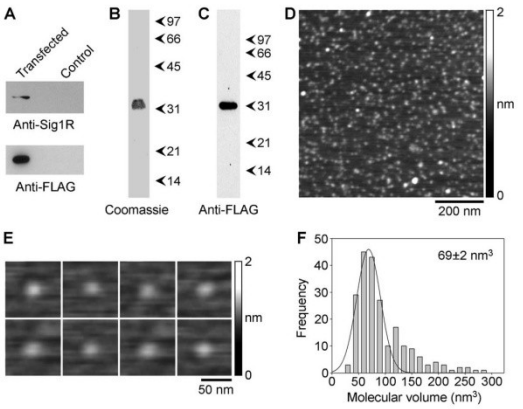

FIGURE 2. **Isolation and AFM imaging of the Sig1R from singly transfected tsA 201 cells.** *A*, detergent extracts of Sig1R-FLAG-transfected and control cells were analyzed by SDS-PAGE followed by immunoblotting using either a mouse monoclonal anti-FLAG antibody (*bottom panel*) or a rabbit polyclonal anti-Sig1R antibody (*top panel*). *B* and *C*, samples of protein isolated by immunoaffinity chromatography on anti-FLAG-agarose were analyzed by SDS-PAGE followed by either Coomassie Blue staining (*B*) or immunoblotting using a mouse monoclonal anti-FLAG antibody (*C*). *Arrowheads* indicate molecular mass markers (kDa). *D*, low-magnification AFM image of a sample of isolated Sig1R. *E*, gallery of enlarged images of Sig1R particles. *F*, frequency distribution of volumes of Sig1R particles. The *curve* indicates the fitted Gaussian function. The peak of the distribution is indicated.

voltage (I_{Na}-V) relationships were constructed as described previously (29). Briefly, from a holding potential of −100 mV the membrane was stepped to potentials between −90 mV and +60 mV, in 5-mV increments, for 50 ms at a frequency of 2 Hz.

RESULTS

tsA 201 cells were transfected with DNA encoding either Sig1R-FLAG, Nav1.5-HA, or both. Protein expression and localization was confirmed by immunofluorescence, using appropriate anti-tag antibodies. As shown in Fig. 1*A*, singly transfected cells gave positive immunofluorescence signals with anti-FLAG (Sig1R-FLAG) or anti-HA (Nav1.5-HA) antibodies, indicating the successful expression of the two proteins. In contrast, an anti-V5 antibody gave only a background signal (data not shown). In doubly transfected cells, the anti-HA and anti-FLAG signals extensively overlapped (Fig. 1*B*), indicating that the majority of transfected cells expressed both proteins. Again, an anti-V5 antibody gave only a background signal (data not shown). The reticular staining patterns suggest that both proteins were localized predominantly in the endoplasmic reticulum.

It is known that HEK-293 cells endogenously express the Sig1R (3). To assess the relative expression levels of endogenous and exogenous (FLAG-tagged) receptors in the tsA 201 cells, detergent extracts of Sig1R-FLAG-transfected and control cells were subjected to immunoblot analysis. The transfected cells gave strong signals with both an anti-FLAG antibody and an anti-Sig1R antibody (Fig. 2*A*). In contrast, the non-transfected cells gave no detectable signal with either antibody. Hence, endogenous Sig1R must be expressed at a much lower level than FLAG-tagged Sig1R and is therefore unlikely to interfere with the subsequent experiments.

Cells expressing Sig1R-FLAG alone were solubilized in Triton X-100 detergent (1% w/v), and the protein was isolated through the binding of the FLAG tag to anti-FLAG-agarose beads, followed by elution with a triple-FLAG peptide. A Coomassie Blue-stained gel of the isolated protein (Fig. 2*B*) shows a single major band at a molecular mass of 33 kDa. The isolated protein was also analyzed by immunoblotting using an anti-FLAG antibody (Fig. 2*C*). A single immunopositive band was seen, again at 33 kDa. This result demonstrates the successful isolation of Sig1R-FLAG from the transfected cells.

Low-magnification AFM images of isolated Sig1R-FLAG revealed a relatively homogenous distribution of particles (Fig. 2*D*). A gallery of zoomed images of individual particles is shown in Fig. 2*E*. The molecular volumes of a number of these particles were calculated, using Scanning Probe Image Processor. A frequency distribution of the volumes had a single peak, at 69 ± 2 (SE) nm^3 ($n = 216$), close to the expected volume of 63 nm^3 for a Sig1R, of a molecular mass of 33 kDa, according to Equation 2 (Fig. 2*F*). Hence, the imaged particles represent individual Sig1Rs.

Protein was next isolated from cells co-expressing Sig1R-FLAG and Nav1.5-HA through the binding of the HA tag on Nav1.5 to anti-HA beads, followed by elution with HA peptide. The isolated sample was analyzed by immunoblotting with

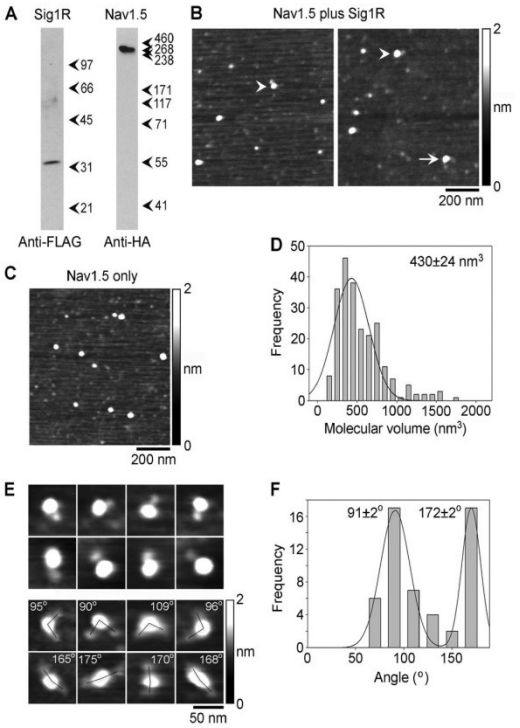

FIGURE 3. **Isolation and analysis of Sig1R-FLAG·Nav1.5-HA complexes by immunoaffinity chromatography on anti-HA agarose.** *A*, samples of protein isolated by immunoaffinity chromatography were analyzed by SDS-PAGE followed by immunoblotting using either mouse monoclonal anti-FLAG (*left panel*) or mouse monoclonal anti-HA antibodies (*right panel*). *Arrowheads* indicate molecular mass markers (kDa). *B*, low-magnification AFM images of samples of isolated Sig1R-FLAG/Nav1.5. Singly and doubly decorated large particles are indicated by *arrowheads* and an *arrow*, respectively. *C*, low-magnification AFM image of Nav1.5 alone, isolated by immunoaffinity chromatography on anti-HA-agarose. *D*, frequency distribution of volumes of large particles isolated from co-transfected cells that were decorated by Sig1R particles. The *curve* indicates the fitted Gaussian function. The peak of the distribution is indicated. *E*, gallery of zoomed images of Nav1.5 channels that were decorated by either one (*upper panels*) or two Sig1Rs (*lower panels*). Angles between pairs of bound Sig1Rs are indicated. *F*, frequency distribution of angles between pairs of bound Sig1Rs. The *curve* indicates the fitted Gaussian functions. The peaks of the distribution are indicated.

anti-FLAG and anti-HA antibodies. The anti-FLAG antibody detected a single band at 33 kDa, as expected for the Sig1R (Fig. 3*A*, *left panel*), and the anti-HA blot revealed a single band at 260 kDa, as expected for Nav1.5 (Fig. 3*A*, *right panel*). The fact that the Sig1R was co-isolated with Nav1.5 indicates an intimate association between the two proteins.

Low-magnification AFM images of co-isolated Sig1R and Nav1.5 showed a population of large particles, some of which were decorated by one (*arrowheads*) or two (*arrow*) smaller particles (Fig. 3*B*). In contrast, corresponding images of protein isolated from cells expressing Nav1.5 alone showed fewer decoration events (Fig. 3*C*). This result suggests that the small bound particles are Sig1Rs and the large particles are Nav1.5 channels.

To quantitate decoration of the Nav1.5 channels by the Sig1Rs, we set a volume range between 30 and 120 nm^3, based on the volume of particles seen with the Sig1R alone (Fig. 2*F*). Of 2482 large particles, 143 (5.8%) were singly decorated by Sig1Rs, and of 6473 particles, 55 (0.8%) were doubly decorated. Corresponding percentages for Nav1.5 alone were as follows: 1.7% (56 of 3,244) singly decorated and 0.1% (7 of 5,917) doubly decorated. Hence, the vast majority of the decoration events seen were specific. A frequency distribution of volumes of the decorated central particles (Fig. 3*D*) had a single peak at 430 ± 24 nm^3 ($n = 231$), close to the expected volume of 490 nm^3 for a Nav1.5 particle with a molecular mass of 260 kDa. This result further supports the suggestion that these particles were indeed Sig1R-decorated Nav1.5 channels.

Galleries of zoomed images of singly and doubly decorated large particles are shown in Fig. 3*E*. We identified Nav1.5 channels that had been decorated by two Sig1Rs and measured the angles between the bound receptors. This was done in each case by joining the highest point on the central particle (the Nav1.5 channel) to the highest points on the peripheral particles (the Sig1Rs) by lines and then determining the angle between the two lines. A frequency distribution of the angles obtained is shown in Fig. 3*F*. The angle distribution has two peaks: a large peak at 91 ± 2° ($n = 34$) and a smaller peak at 172 ± 2° ($n = 19$); the ratio of the numbers of particles in the two peaks is 1.8:1. This angle profile, with two peaks at ~90° and 180°, in a ratio of ~2:1 suggests that the Nav1.5 presents four perpendicular binding sites to the Sig1R and that these are randomly occupied.

To further characterize the interaction between Sig1R and Nav1.5, we isolated protein from co-transfected cells through the binding of the FLAG tag on the Sig1R to anti-FLAG agarose, followed by elution with the triple-FLAG peptide. The isolated protein was analyzed by immunoblotting. As shown in Fig. 4*A*, the anti-FLAG blot showed a band at 33 kDa (*left panel*), and the anti-HA blot showed a band at 260 kDa (*right panel*). These results are very similar to those obtained after purification through the HA tag on Nav1.5 and support our assertion that the Sig1R and Nav1.5 interact intimately within the cells.

Given that the immunofluorescence images (Fig. 1) indicated that the majority of exogenously expressed protein was intracellular, whereas Nav1.5 clearly functions at the plasma membrane, we felt that it was important to establish whether the Sig1R and Nav1.5 interacted at the plasma membrane. To do this, we biotinylated proteins in the plasma membrane of intact co-transfected cells before preparing a detergent extract. Biotinylated proteins in the extract were captured on monomeric avidin-agarose and then eluted using free biotin. Biotinylated Sig1R-FLAG was then recaptured using anti-FLAG-agarose, followed by elution with triple-FLAG peptide. As shown in Fig. 4*B*, immunoblotting detected both Sig1R-FLAG and Nav1.5-HA in the final eluate, demonstrating an interaction between the two proteins when at least one of them was in the plasma membrane. To control for cell lysis during biotinylation, immunoblotting for β-actin was carried out; β-actin was detected in the total extract but not in the eluate from the avidin-agarose (data not shown), indicating that there was no significant cell lysis.

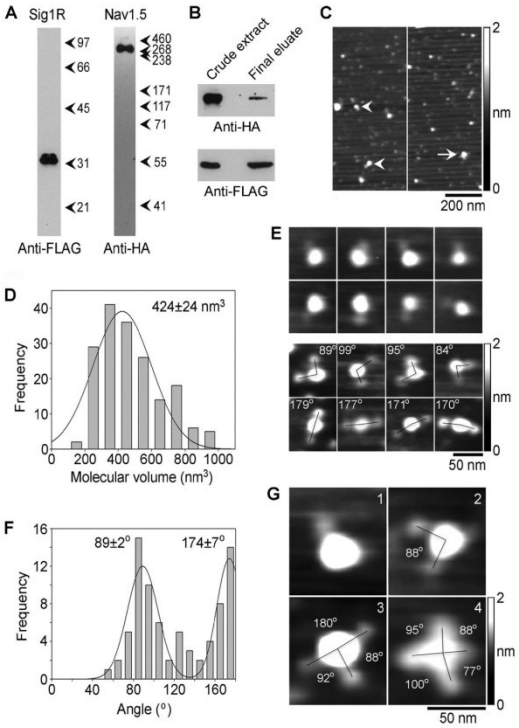

FIGURE 4. **Isolation and analysis of Sig1R-FLAG·Nav1.5-HA complexes by immunoaffinity chromatography on anti-FLAG-agarose.** *A*, samples of protein isolated by affinity chromatography were analyzed by SDS-PAGE followed by immunoblotting using either mouse monoclonal anti-FLAG (*left panel*) or mouse monoclonal anti-HA antibodies (*right panel*). *Arrowheads* indicate molecular mass markers (kDa). *B*, a sample of protein isolated from surface-biotinylated, intact co-transfected cells by sequential affinity chromatography on monomeric avidin-agarose and anti-FLAG-agarose was analyzed by SDS-PAGE followed by immunoblotting using either mouse monoclonal anti-FLAG (*bottom panel*) or mouse monoclonal anti-HA antibodies (*top panel*). A crude detergent extract of the cells (at a loading ratio of 1:128 compared with the final eluate) was also analyzed. *C*, low-magnification AFM images of samples of isolated Sig1R-FLAG/Nav1.5. Singly- and doubly-decorated large particles are indicated by *arrowheads* and an *arrow*, respectively. *D*, frequency distribution of volumes of large particles that were decorated by Sig1R particles. The curve indicates the fitted Gaussian function. The peak of the distribution is indicated. *E*, gallery of enlarged images of Nav1.5 channels that were decorated by either one (*upper panels*) or two Sig1Rs (*lower panels*). Angles between pairs of bound Sig1Rs are indicated. *F*, frequency distribution of angles between pairs of bound Sig1Rs. The curve indicates the fitted Gaussian functions. The peaks of the distribution are indicated. *G*, gallery of zoomed images of Nav1.5 particles decorated by one, two, three, or four Sig1R particles. Angles between pairs of bound Sig1Rs are indicated.

Low-magnification AFM images of protein isolated from a total cell extract showed a population of large particles some of which were decorated by either one (*arrowheads*) or two (*arrow*) smaller particles (Fig. 4*C*). In contrast, as shown above in Fig. 2*D*, an image of proteins isolated from cells singly transfected with the Sig1R alone shows very few larger particles.

As above, small bound particles in the volume range 30–120 nm^3 were accepted as Sig1Rs. A frequency distribution of molecular volumes of the large decorated particles was then constructed. As shown in Fig. 4*D*, the distribution has a single peak at 424 ± 24 nm^3 (n = 177), close to the value obtained for particles isolated through the HA tag on Nav1.5 (430 nm^3, Fig. 3*D*) and to the value of 490 nm^3 expected for a protein of molecular mass 260 kDa.

Galleries of zoomed images of singly- and doubly-decorated large particles are shown in Fig. 4*E*. A frequency distribution of angles between pairs of bound Sig1Rs (Fig. 4*F*) had two peaks, at 89 ± 2° (n = 49) and 174 ± 7° (n = 28), and the ratio of the sizes of the two peaks was 1.7:1, close to the values obtained in the reverse purification (91° and 172°, Fig. 3*F*) and to the predicted value of 2:1 for random decoration of a protein with 4-fold symmetry. Rarely, we saw Nav1.5 particles that were decorated by three (n = 4) or four (n = 2) Sig1R particles. Fig. 4*G* shows a gallery of zoomed images of Nav1.5 particles decorated by one, two, three, or four Sig1R particles. This gallery nicely illustrates the central conclusion of our study.

A number of Sig1R ligands are known to affect the function of ion channels, including Nav1.5 (3, 4, 23). We have shown previously that the ligand haloperidol reduced decoration of ASIC1a by the Sig1R by ~50% (27). We therefore decided to test the effects of two Sig1R ligands, haloperidol and (+)-pentazocine, on the ability of Sig1R to capture Nav1.5 from a detergent extract of co-transfected cells. Drugs were incubated with the detergent extracts for 1 h at 4 °C, before the extracts were added to the immunobeads. As shown in Fig. 5*A*, neither ligand had any effect on the binding of the Sig1R to anti-FLAG agarose; however, both reduced the co-isolation of Nav1.5. The intensity of the Nav1.5 band was reduced to 80% of control by haloperidol and to 10% of control by (+)-pentazocine. Hence, both ligands reduce the interaction between the Sig1R and Nav1.5 when added *in vitro*.

To establish whether haloperidol and (+)-pentazocine also reduce the interaction between Nav1.5 and Sig1R within the tsA 201 cells, *in situ* proximity ligation assays were carried out (36). The assay uses two secondary antibodies, each bearing a short DNA strand. When the secondary antibodies are brought into close proximity (<40 nm) by binding to their relevant primary antibodies, the DNA strands hybridize with an additional circle-forming oligodeoxynucleotide. Ligation then creates a complete circularized oligodeoxynucleotide, and rolling circle amplification increases the amount of circular DNA several hundredfold. The DNA is then visualized using a fluorescent probe. Cells were co-transfected with Nav1.5-HA and Sig1R-FLAG, and drugs were added to the medium 1 h before the cells were fixed. Primary antibodies used to tag the subunits were rabbit anti-HA (Nav1.5) and mouse anti-FLAG (Sig1R). As shown in Fig. 5*B*, a strong proximity signal was seen in the control sample, indicating that Nav1.5 and Sig1R come into close proximity within the co-transfected cells. The intensity of the proximity signal was reduced by both haloperidol and (+)-pentazocine, with the latter drug having the greater effect. Hence, the two ligands have similar effects on the Nav1.5/Sig1R interaction *in vitro* and in intact cells.

The experiments described thus far have used overexpressed, epitope-tagged Nav1.5 and Sig1R. We were therefore interested to examine whether Nav1.5 and Sig1R would also interact when expressed endogenously and whether such an interaction would have functional consequences for Nav1.5

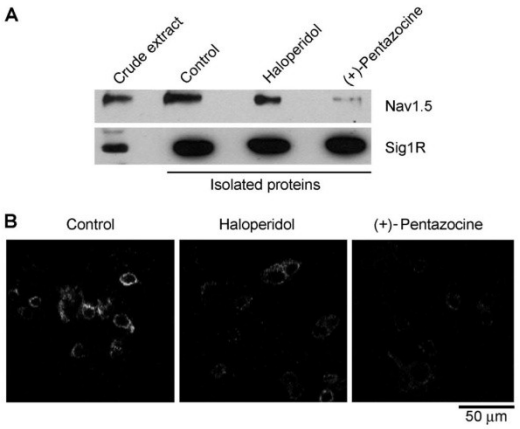

FIGURE 5. **Effect of Sig1R ligands on the interaction between the Sig1R and Nav1.5.** *A*, the Sig1R was isolated from Sig1R-FLAG/Nav1.5-HA co-transfected cells by immunoaffinity chromatography on anti-FLAG agarose. The Sig1R ligands haloperidol (Sigma; 100 μM) and (+)-pentazocine (Sigma; 10 μM) were incubated with identical samples of a crude detergent extract of the cells for 1 h at 4 °C before addition to the immunobeads. Samples of isolated protein were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using either mouse monoclonal anti-HA (*top panel*) or anti-FLAG antibodies (*bottom panel*). The immunoblots shown are representative of the results from three separate experiments. A crude detergent extract of the cells (at a loading ratio of 1:32 compared with the final eluate) was also analyzed. *B*, cells were co-transfected with DNA encoding Nav1.5-HA and Sig1R-FLAG. Haloperidol (100 μM) and (+)-pentazocine (10 μM) were added to the media for 1 h before the cells were fixed. Cells were permeabilized and incubated with primary antibodies (rabbit polyclonal anti-HA and mouse monoclonal anti-FLAG) followed by anti-mouse (+) and anti-rabbit (−) proximity ligation secondary antibodies. The proximity ligation assay was then carried out and treated cells were imaged by confocal laser scanning microscopy. All images were captured using identical microscope settings. *Scale bar*, 50 μm.

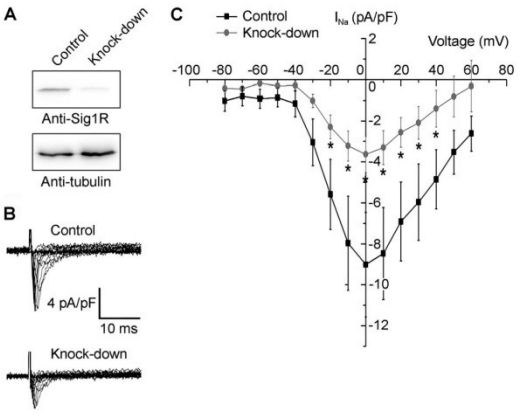

FIGURE 6. **Sig1R expression regulates Nav1.5 current density in MDA-MB-231 cells.** *A*, immunoblot showing the shRNA-induced knock-down of Sig1R expression in MDA-MB-231 cells. α-Tubulin is shown as a control (antibody from Sigma). *B*, representative Na^+ currents elicited in Sig1R knockdown or control MDA-MB-231 cells by a series of depolarization pulses between +60 and −80 mV from a holding potential of −100 mV. *C*, current-voltage relationship of the Na^+ current obtained from a holding potential of −100 mV (n = 10 cells). *, $p < 0.05$, Mann-Whitney test.

channel behavior. To do this, we recorded Nav1.5 currents in control MDA-MB-231 cells and in cells in which Sig1R expression had been knocked down using shRNA; knockdown was confirmed by immunoblotting (Fig. 6*A*). The membrane potential was depolarized from a holding potential at −100 mV to potentials between −90 and +60 mV. A strong reduction of voltage-dependent Na^+ current was observed in Sig1R knockdown cells compared with control cells (Fig. 6, *B* and *C*). Specifically, at 0 mV, the maximal current amplitude was −3.63 ± 0.84 pA/pF in Sig1R-silenced cells, and −9.01 ± 2.54 pA/pF in control cells (n = 10 cells for each condition). Thus, the knockdown of Sig1R expression in MDA-MB-231 cells reduces the Nav1.5 current by ~60%, demonstrating that the Sig1R regulates Nav1.5 function in these cells.

DISCUSSION

In the present study, we have shown that the Sig1R binds to Nav1.5, which contains four six-TMR cassettes, with 4-fold symmetry. In a previous study, we used a similar approach to demonstrate that the Sig1R binds to the trimeric ASIC1a ion channel with 3-fold symmetry (27). Of course, the fact that Nav1.5 contains all four TMR cassettes in a single polypeptide means that an intact channel has only one N- and one C- terminal domain. This would appear to rule out the possibility that either of these domains is involved in Sig1R binding, suggesting instead the likelihood that the Sig1R binds to the TMRs of its target proteins. This suggestion is supported by a recent study, which showed that the Sig1R could be co-immunoprecipitated with a truncation mutant of Kv1.3 that was essentially pared down to its TMRs (9).

If the Sig1R does indeed bind to the TMRs of its targets, the interaction is likely to be hydrophobic. There is no obvious structural motif shared by all Sig1R targets, and the Sig1R itself contains no known protein interaction motifs, such as SH3, PDZ, or WW domains (5). Significantly, two so-called sterol binding-like domains have been identified in the Sig1R (38, 39). One of these domains (residues 91–109) encompasses part of the second transmembrane domain, and the other (residues 176–194) forms a hydrophobic region close to the C terminus. The C-terminal hydrophobic region contains cholesterol binding domain motifs (VEYGR and LFYTLRSYAR), and as expected, this region binds cholesterol (40). Consistent with an involvement of cholesterol in the interaction between the Sig1R and its ion channel targets, both the Sig1R itself (18, 40) and ion channels such as Nav1.5 (31), Kv1.4 (41), and ASIC3 (42) are localized to cholesterol-enriched membrane regions, sometimes known as lipid "rafts." The Sig1R ligand SKF-10047 has been shown to strongly inhibit cholesterol binding to the Sig1R and at the same time to reduce the raft localization of the receptor (40), suggesting that the cholesterol-binding domain forms part of the drug binding site. The observed effects of the two Sig1R ligands used here on the interaction between the Sig1R and Nav1.5 suggest that the action of the drugs might be mediated through displacement of cholesterol from the Sig1R. This possibility now needs to be tested in further studies.

Only a small proportion of the Nav1.5 particles isolated from cells co-expressing Nav1.5 and the Sig1R (~6%) was decorated by Sig1Rs, and most decorated channels had only a single asso-

ciated Sig1R. This result could indicate that the Sig1R interacts with only a subpopulation of the Nav1.5 channels within the cell, possibly at a particular stage of the intracellular transport pathway. Alternatively, the interaction between Nav1.5 and the Sig1R could be transient and/or sensitive to the addition of detergent used in the isolation protocol. The robust signal seen in the proximity ligation assay indicates that the two proteins do interact in the intact cell. Furthermore, the surface biotinylation experiment shows that the proteins interact when at least one of them is present in the plasma membrane. However, the stoichiometry of the interaction between the Sig1R and Nav1.5 in intact cells remains unresolved.

It has been reported previously that Nav1.5 promotes the invasiveness of the aggressive breast cancer cell line, MDA-MB-231 (29–31). Furthermore, the Sig1R was also found to be overexpressed in this cell line compared with a normal epithelial breast cell line (20, 40). Here, we have shown that Sig1R expression regulates Nav1.5 function in MDA-MB-231 cells. A similar observation was reported recently for the Sig1R regulation of the maturation and membrane stability of the heterotetrameric K^+ channel, hERG, in leukemia cells (11), although further studies are needed to determine whether Sig1R regulates hERG and Nav1.5 through a common mechanism.

Voltage-gated Na^+ channels such as Nav1.5 contain a pore-forming α-subunit and one or more β-subunits (28). Most of the experiments described here involved overexpression of the α-subunit in a cell line (tsA 201) that is unlikely to express a β-subunit. Consequently, we cannot completely rule out the possibility that the presence of a β-subunit might affect the interaction between the Sig1R and Nav1.5. However, we were able to observe a functional interaction between endogenous Sig1R and Nav1.5 in MDA-MB-231 cells, which are known to express Nav1.5 β-subunits (43). It is clear, therefore, that the Sig1R/Nav1.5 interaction does occur in the presence of the β-subunits.

In conclusion, we have demonstrated a direct interaction between the Sig1R and Nav1.5, revealed the architecture of the Sig1R·Nav1.5 complex, and shown that this interaction has functional consequences for Nav1.5 in a cancer cell line.

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3. Discussion :

Sig1R peut agir directement ou indirectement sur les canaux ioniques (III.E). Au cours de la première étude de ma thèse, nous avons montré par co-immunoprécipitation, que Sig1R était associé à hERG (Crottes et al., 2011).

Avec la même méthodologie, il a été observé que Sig1R s'associe aux canaux K_v1.4, K_v1.2, K_v1.3, IP3-R et Ca_v de type L (Abou-Lovergne et al., 2011; Aydar et al., 2004; Hayashi and Su, 2001, 2007; Kinoshita et al., 2012; Kourrich et al., 2013; Tchedre et al., 2008; Wu and Bowen, 2008) (III.E). Cependant, cette technique ne permet pas d'expliquer comment Sig1R interagit avec ces canaux ioniques. Pour élucider les caractéristiques de cette interaction, des expériences de mutagenèse ont révélé que la partie C-terminale de Sig1R est responsable de l'interaction de Sig1R avec le canal IP3-R3 (Wu and Bowen, 2008) et que Sig1R interagit avec les domaines transmembranaires du canal K_v1.3 (Kinoshita et al., 2012).

Dans le même temps, une série d'études menées par l'équipe du Pr. Mike Edwardson a permis de mettre en évidence la relation entre Sig1R et les canaux ioniques en utilisant la technique de microscopie de force atomique. Cette technique permet de caractériser une interaction directe entre deux protéines et de mesurer à la fois la stœchiométrie et la géométrie de l'interaction. Ainsi, Sig1R s'associe directement avec le canal ASIC1a (canal cationique sensible au pH extracellulaire) et le récepteur NMDA (Balasuriya et al., 2013; Carnally et al., 2010). Ces canaux ioniques s'associent respectivement sous forme de trimères et de dimères. Chaque sous-unité α fixe potentiellement une seule protéine Sig1R. La géométrie de ces interactions indique que Sig1R est très probablement relié au domaine transmembranaire de chaque sous-unité α.

En collaboration avec l'équipe du Pr. Mike Edwardson, nous avons montré par microscopie de force atomique que Sig1R interagit directement avec le canal sodique Na_v1.5. Puis, nous avons déterminé que cette interaction suit une stœchiométrie de quatre protéines Sig1R par canal Na_v1.5. Cela suggère que Sig1R interagit avec un motif présent en quatre exemplaires sur la sous-unité α du canal Na_v1.5. Ce dernier se compose d'une sous-unité α composée de quatre domaines transmembranaires et de deux extrémités N- et C-terminales (Figure 8). Ainsi, il est raisonnable de proposer que Sig1R interagit avec les domaines transmembranaires du canal Na_v1.5 plutôt qu'avec ces extrémités N- et C-terminales.

Nos résultats confirment donc ceux obtenus précédemment par l'équipe de Pr. Mike Edwardson et tendent à prouver que Sig1R interagit directement avec les canaux ioniques en se fixant sur les domaines transmembranaires des sous-unités α des canaux ioniques. Des expériences préliminaires menées en collaboration avec l'équipe du Pr. Mike Edwardson montrent que Sig1R s'associe également au canal hERG de façon directe et suivant la même stœchiométrie (une protéine Sig1R par monomère de hERG) (Annexe 1).

Ainsi, nos résultats et ceux de la littérature indiquent que Sig1R s'associe directement avec certains canaux ioniques en utilisant leurs domaines transmembranaires comme motif de reconnaissance. Ceci explique pourquoi Sig1R est capable d'agir sur une aussi grande diversité de canaux ioniques sans que ne soit identifiée de séquence consensus.

Cependant quelques zones d'ombres persistent. En effet, si Sig1R interagit avec les canaux ioniques à travers le (ou les) domaine(s) transmembranaire(s) des sous-unités α, comment expliquer le fait que Sig1R interagit avec la sous-unité GluN1 du récepteur au NMDA et pas avec son homologue GluN2 (Balasuriya et al., 2013)? Avec l'IP3-R3 et R1 et pas l'IP3-R2 (Abou-Lovergne et al., 2011) ? Cela laisse imaginer qu'il existe des différences minimes entre ces homologues qui conditionnent leur liaison à Sig1R. A l'heure actuelle, aucune étude n'a mis en évidence ces différences.

Par ailleurs, s'il existe une homogénéité de l'interaction de Sig1R avec les canaux ioniques, comment expliquer l'hétérogénéité de son action ? On peut envisager qu'il existe une variabilité dans les protéines ou dans les éléments interférants avec le complexe Sig1R / canaux ioniques, soutenant ainsi notre idée de Sig1R en tant « qu'adaptateur universel » proposée lors de notre première étude (V.A.3).

Notre étude sur le canal $Na_v1.5$ a également permis d'établir que l'inhibition de l'expression de Sig1R réduit le courant sodique généré par le canal $Na_v1.5$ dans les cellules cancéreuses mammaires MDA-MB-231. Dans les cancers du sein, $Na_v1.5$ s'associe avec l'échangeur sodium/proton, NHE1 localisé dans des structures très spécialisées, les invadopodes. Dans ces structures, l'activité de $Na_v1.5$ permet de soutenir celle de NHE1, d'acidifier le milieu extracellulaire et de favoriser la dégradation de la matrice extracellulaire par les cathepsines B. A travers ce mécanisme, le canal $Na_v1.5$ soutient l'invasion de ces cellules cancéreuses (Brisson et al., 2011).

Pour compléter cette étude, il serait particulièrement intéressant d'observer le rôle de l'expression de Sig1R sur la formation du complexe $Na_v1.5$ / NHE1 et l'activité des invadopodes. Dans ce sens, des travaux sont actuellement en cours.

VI. Conclusion et Perspectives :

En résumé, au cours de ma thèse, j'ai identifié comment Sig1R régulait l'activité du canal potassique voltage-dépendant, hERG. J'ai ainsi démontré que Sig1R module hERG en agissant sur sa maturation, sa stabilité et son expression à la membrane plasmique. Ces observations soutiennent l'idée que Sig1R agit sur les canaux ioniques de multiples façons.

Dans un deuxième temps, nous avons observé l'interaction directe entre Sig1R et le canal $Na_v1.5$. Notre étude s'inscrit dans la continuité des études du Pr. Mike Edwardson et permet de renforcer le concept que Sig1R interagit avec les canaux ioniques en se fixant sur les domaines transmembranaires de chaque monomère constituant la sous-unité α des canaux ioniques.

Tout ceci nous a amené à proposer Sig1R comme un « adaptateur universel ». Sig1R s'associerait préférentiellement avec les canaux ioniques à travers la reconnaissance d'un motif contenu dans leurs domaines transmembranaires et permettrait ainsi de réguler leur activité en fonction des protéines interagissant avec le complexe Sig1R / canal ionique.

J'ai vérifié cette hypothèse en identifiant le rôle de Sig1R dans la plasticité électrique induite par la MEC. Lors de l'interaction de la cellule cancéreuse avec la MEC, la présence de Sig1R favorise l'activité ionique du canal hERG dépendante de l'activation de la sous-unité β1 des intégrines par la MEC en augmentant l'expression à la surface de canaux hERG et leur association avec la sous-unité β1 des intégrines. Malgré cela, il convient de valider cette hypothèse en identifiant un mécanisme similaire de Sig1R dans la formation d'autres complexes protéiques impliquant des canaux ioniques (par exemple, hERG / Flt-1, hERG / CXCR4 ou $Na_v1.5$ / NHE1).

Le rôle de Sig1R dans le développement tumoral n'a été que très peu étudié. Néanmoins, il ressort de la littérature que l'expression de Sig1R est plus importante dans les cellules cancéreuses que dans des cellules saines (II.I). Ici, nos analyses d'expériences de génomique confirment cela. Nos résultats indiquent que l'inhibition de l'expression de Sig1R exerce un effet basal sur l'activité des courants ioniques générés par les canaux hERG et $Na_v1.5$. Les travaux précédents du laboratoire observent également un effet de l'inhibition de l'expression de Sig1R sur l'activité de courant ionique indépendamment de la présence de ligands (Renaudo et al., 2007). Ces observations suggèrent donc une activité « constitutive » de Sig1R dans les cellules cancéreuses. L'activité de Sig1R étant décrite comme dépendante du stress cellulaire, cela signifie-t-il que les cellules cancéreuses sont constitutivement soumises à des situations stressantes ? Depuis quelques années, il a été

observé que l'environnement des cellules tumorales se caractérise par des zones hypoxiques, des zones de nécroses et une privation en nutriments. Ces conditions engendrent des stress cellulaires chroniques (Hetz et al., 2013; Moenner et al., 2007). Il est possible alors que ces stress chroniques puissent activer Sig1R de façon constitutive. L'expression de Sig1R serait donc bénéfique aux cellules cancéreuses afin de les adapter à cet environnement stringent. Cependant, des études complémentaires seront nécessaires afin d'identifier la relation entre Sig1R et le stress dans le contexte tumoral.

Notre étude démontre également l'importance de l'expression de Sig1R dans la plasticité électrique des cellules cancéreuses et la progression tumorale en lien avec leur interaction avec un élément du microenvironnement tumoral, la matrice extracellulaire (MEC). En effet, nous observons grâce à des approches *in vitro* et *in vivo* que l'expression de Sig1R participe à de multiples fonctions pro-tumorales (migration, angiogenèse, extravasation) et favorise ainsi le potentiel invasif des cellules cancéreuses. Cela suggère que Sig1R est potentiellement un élément clé du dialogue tumeur / microenvironnement. D'autres études seront nécessaires afin de valider le rôle de Sig1R dans les interactions qu'entretiennent les cellules cancéreuses avec les autres acteurs du microenvironnement tumorales (fibroblastes, cellules immunes, …)

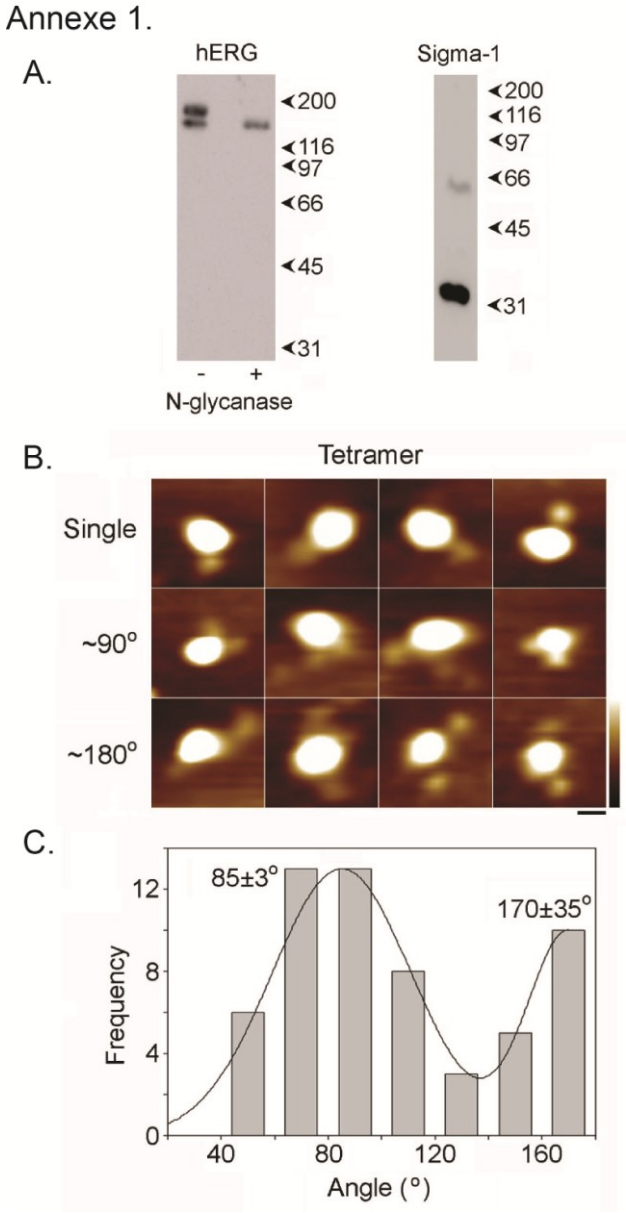
Mes travaux devraient donc permettre de valider le rôle de Sig1R dans les cancers et de proposer cette protéine chaperonne comme un biomarqueur ou une cible thérapeutique potentielle.

Cibler Sig1R dans les cancers présente potentiellement des avantages d'un point de vue thérapeutique. Les études menées dans le tissu cérébral indiquent nettement que Sig1R n'est actif que dans des tissus en état de stress et n'a pas d'activité dans des conditions normales (Su et al., 2010). Dans les cancers, la seule expression de Sig1R a un rôle important dans la progression tumorale et favorise le potentiel invasif des cellules cancéreuses. Mieux, la plupart des agonistes de Sig1R ont un effet neuroprotecteur. Or dans les cancers, ces mêmes agonistes montrent un effet inhibiteur sur les fonctions des cellules cancéreuses.

Ainsi, cibler Sig1R pour inhiber les fonctions des canaux ioniques dans les cancers est une stratégie plus intéressante que de cibler les canaux ioniques eux-mêmes. Cette stratégie ciblant l'activité de Sig1R dans les cellules cancéreuses pourrait se révéler efficace pour réduire la progression tumorale et devrait présenter que peu de risques secondaires pour le patient.

De plus amples études seront cependant nécessaires afin de confirmer nos différentes hypothèses (protéine adaptatrice, protéine de réponse au microenvironnement tumoral) sur le rôle moléculaire de Sig1R mais aussi pour étendre le rôle de Sig1R à d'autres canaux ioniques et d'autres cancers.

Annexes :



Annexe 1 : Sig1R interagit directement avec le canal hERG avec une stœchiométrie de 1 :1. Les séquences de Sig1R et hERG fusionnées avec respectivement un tag « c-myc » et « HA » sont stablement exprimés dans des cellules HEK293. Les protéines sont extraites par une technique de chromatographie d'immunoaffinité en utilisant des anticorps anti-cmyc et anti-HA. A. Immunoblot montrant l'expression des protéines hERG (sous ses différentes formes de maturation) et Sig1R dans le modèle cellulaire HEK293. B. Les échantillons protéiques obtenus sont déposés sur une plaque de mica avant d'être visualisés par un microscope de force atomique (Veeco Digital Instruments Multimode AFM). Image représentative montrant un tétramère de hERG « décoré » par plusieurs protéines Sig1R. C. Les images obtenues sont analysées par un logiciel de calcul de volumes moléculaires (Scanning Probe Image Processor, Image Metrology version 5). Ici est reportés l'angle formé par plusieurs protéines Sig1R sur un tétramères de hERG. (Pour plus d'information sur la méthodologie de cette expérience : Carnally et al., 2008).

Annexe 2 : The sigma-1 receptor : a regulator of cancer cell electrical plasticity

frontiers in PHYSIOLOGY

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The sigma-1 receptor: a regulator of cancer cell electrical plasticity?

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Originally mistaken as an opioid receptor, the sigma-1 receptor (Sig1R) is a ubiquitous membrane protein that has been involved in many cellular processes. While the precise function of Sig1R has long remained mysterious, recent studies have shed light on its role and the molecular mechanisms triggered. Sig1R is in fact a stress-activated chaperone mainly associated with the ER-mitochondria interface that can regulate cell survival through the control of calcium homeostasis. Sig1R functionally regulates ion channels belonging to various molecular families and it has thus been involved in neuronal plasticity and central nervous system diseases. Interestingly, Sig1R is frequently expressed in tumors but its function in cancer has not been yet clarified. In this review, we discuss the current understanding of Sig1R. We suggest herein that Sig1R shapes cancer cell electrical signature upon environmental conditions. Thus, Sig1R may be used as a novel therapeutic target to specifically abrogate pro-invasive functions of ion channels in cancer tissue.

Keywords: sigma-1 receptor, chaperones, ion channels modulation, stress, physiological, cancer

INTRODUCTION

The concept of "Sigma receptors" arose 40 years ago in a pharmacological study postulating the existence of three types of opioid receptors on the basis of the psychomimetic effects induced by several opioid compounds (μ, κ and σ receptor respectively accounting for the effects produced by morphine, ketacyclazocine and SKF 10,047) (Martin et al., 1976). Further pharmacological studies revealed the existence of two binding sites, namely Sigma 1 (Sig1R) and Sigma 2 receptors (Sig2R) (Quirion et al., 1992). The Sig1R was cloned in 1996 (gene names: SIGMAR1 or OPRS1) and the gene is located on 9p13 (Hanner et al., 1996; Prasad et al., 1998). Sig1R is a 25-kDa protein anchored in the endoplasmic reticulum (ER) with no similarity with other known mammalian proteins, thus definitely ruling out any connection with a classical receptor family. Sig1R possesses two transmembrane regions and two steroid binding domains (SBD). These domains form a pocket which is the binding site for cholesterol, steroids, sphingolipids (Palmer et al., 2007; Fontanilla et al., 2008), and also for a wide panel of synthetic or natural compounds (sigma ligands) from different classes such as opioids, antipsychotics, psychostimulants, alkaloids or antidepressants (Pal et al., 2008; Maurice and Su, 2009) (**Figure 1**). *In vivo*, endogenous dimethyl tryptamine (DMT) interacts with Sig1R in the brain but its physiological significance as an endogenous sigma ligand is not yet clarified (Fontanilla et al., 2009; Mavlyutov et al., 2012).

The molecular nature of Sig2R has long been questioned. A recent work proposed the progesterone receptor membrane component 1 (Pgrmc1) as the putative sigma 2 binding site (Xu et al., 2011). This cytochrome-related protein binds several P450 proteins and various chemical compounds and it participates to cholesterol synthesis. However, while this putative Sig2R shares some pharmacological properties with Sig1R, the two proteins belong to distinct families. This review will focus on Sig1R.

Sig1Rs have been associated with many diseases including stroke, cocaine addiction, Alzheimer's disease, amnesia, amyotrophic lateral sclerosis, retinal degeneration, and cancer (Romieu et al., 2004; Aydar et al., 2006; Renaudo et al., 2007; Maurice and Su, 2009; Luty et al., 2010; Mavlyutov et al., 2011; Ruscher et al., 2011; Kourrich et al., 2013). Nonetheless, the way Sig1R operates in such diseases is still poorly understood. Su and colleagues'work on neurons and CHO cells have shed light on the molecular mechanisms underlying Sig1R functions. Sig1R is mainly located at the ER, in close contact with the mitochondria, in the so-called mitochondria-associated-ER membrane domains (MAM). In resting condition, Sig1R resides in ceramide- and cholesterol-rich lipid microdomains associated with the ER-resident chaperone GRP78 (BiP) (Hayashi and Su, 2007; Hayashi and Fujimoto, 2010). Under cellular stress leading to ER injury, Sig1R dissociates from BiP and binds IP_3 receptors, enhancing in turn cell survival through the control of calcium signaling between the ER and mitochondria. In addition, Sig1R translocates to other cell compartments and binds to different membrane proteins. The stimulation with sigma "agonists" mimicks stress-induced Sig1R dissociation from BiP and Sig1R delocalization, while sigma ligands classified as "antagonists" impede this process (Hayashi and Su, 2007). Altogether, these results have led to a model in which Sig1R is "silent" in normal physiological conditions, whereas in case of a disease, Sig1R behaves as a chaperone that binds client protein to the benefit of cell survival (Su et al., 2010). This exciting hypothesis has been validated by recent studies demonstrating that Sig1R molecular silencing reduces brain

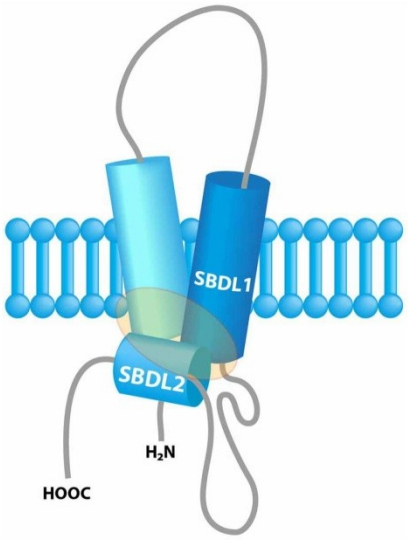

FIGURE 1 | Model of the sigma-1 receptor binding region from previous photolabeling studies. The shaded area represents the ligand binding region. Adapted with permission from Chu et al. (2013).

recovery after experimental stroke (Ruscher et al., 2011) and promotes retina degeneration after acute damage to the optic nerve (Mavlyutov et al., 2011).

The question of client proteins targeted by Sig1R is of importance. Beyond the coupling with IP3 receptors, a number of studies mainly based on the effects of exogenous sigma ligands have shown that Sig1R interferes with dopamine and acetylcholine systems and modulates the function of ion channels belonging to various families. Recent studies have described a molecular interaction between Sig1R and ion channels, suggesting that ion channels represent a major client protein family for the Sig1R chaperone (Carnally et al., 2010; Crottes et al., 2011; Balasuriya et al., 2012; Kourrich et al., 2012). Over the past decades, ion channels have been integrated to the main cellular processes underlying the hallmarks of cancer: tumors often express ion channels and transporters that are absent from the corresponding tissue. It is suggested that these channels and transporters enhance the cell's capacity to adapt themselves to restraint metabolic conditions encountered within the tumor tissue (low pH and PO_2, poor nutrient supply, etc. . .) (Wulff et al., 2009; Prevarskaya et al., 2010; Arcangeli, 2011). Transport proteins therefore participate to the adaptive cancer cells'response to environmental stress, conferring them with greater aggressiveness. This review attempts to draw together the knowledge about ion channel regulation by Sig1R and the recent discoveries on the function of ion channels in cancer. We suggest that upon environmental challenging conditions within the tumor, Sig1R may participate in the electrical remodeling of cancer cell electrical properties to enhance their survival and aggressiveness.

SIGMA 1 RECEPTORS IN CANCER

Binding experiment studies realized in the 90's revealed that sigma receptors are highly expressed in many human and rodent tumor cell lines including breast, lung, prostate, colon, melanoma, neuroblastoma and glioma (John et al., 1995; Vilner et al., 1995b; Aydar et al., 2004). However, most of the sigma ligands used in these studies are not selective enough between Sig1R and Sig2R to draw a definitive conclusion on the density of each binding site in the explored cancer cell types. Using a specific Sig1R antibody, a high expression of Sig1R was found in lung, breast and prostate cancer cell lines whereas low levels were found in normal counterpart cells. Interestingly, the Sig1R density was increased in high metastatic potential cancer cells suggesting a link between Sig1R expression and aggressiveness (Aydar et al., 2006). In another study, the expression of Sig1R was explored by imunohistochemistry in 58 breast cancer patients and 51 normal breasts. Sig1R positive epithelial cell staining was detected in 60 or 41% of invasive or *in situ* cancers respectively, in 75% of ductal hyperplasia and in 33% of normal breast (Wang et al., 2004). Accordingly, scintigraphy with a moderately-selective Sig1R ligand (*N*-[2-(1′-Piperidinyl) Ethyl]-3-^{123}I-Iodo-4-Methoxybenzamide) on patients with primary breast cancer revealed that the ligand was specifically retained within the tumor site, but not in healthy tissues (Caveliers et al., 2001). Several reports indicate that the use of Sig1R ligands to target therapeutic nanoparticles dramatically enhances the delivery of siRNA or drugs at the tumor site in melanoma, prostate, lung and breast cancer (Li and Huang, 2006; Chen et al., 2010; Guo et al., 2012; Kim and Huang, 2012).

Altogether, these studies strongly suggest that Sig1R is over expressed in many cancer cells and an extensive exploration of Sig1R expression in biopsies from various cancers is now required to determine whether Sig1R could be proposed as a diagnosis or prognosis marker.

The effects of sigma ligands on cancer cells' behavior have been assessed by many groups *in vitro* and *in vivo*. Early descriptive works showed that cell treatment with sigma ligands causes rounding, detachment and growth inhibition of C6 glioma (Vilner et al., 1995a), breast and colon carcinoma and melanoma cells (Brent and Pang, 1995; Aydar et al., 2004). Further works by Spruce and coll. showed that the moderately selective ligand rimcazole provokes *in vitro* and *in vivo* (mouse xenograft model) a tumor-selective, caspase-dependent apoptosis of breast and colon cancer (Spruce et al., 2004; Achison et al., 2007). Rimcazole was shown to antagonize a Sig1R-dependent mechanism involving a calcium-dependent activation of phospholipase C, a calcium-independent inhibition of phosphatidylinositol 3′-kinase pathway signaling and the accumulation of HIF-1α. While Sig1R agonists (+) pentazocine and (+) SKF10,047 had no effect *per se*, these ligands could abrogate rimacazole-induced apoptosis, suggesting that in cancer cells, Sig1R is in an activated state and enhances survival. In agreement with this hypothesis, transfection of Sig1R in

HEK293 cells reverses apoptosis induced by the over-expression of Bax or by staurospaurine (Spruce et al., 2004; Achison et al., 2007; Crottes et al., 2011). However, whether Sig1R protects cancer cells from death through a chaperoning activity has not yet been addressed.

Sig1R has also been connected to cell/matrix interaction. Aydar et al. have demonstrated that in breast cancer cells, Sig1R is associated with β1 integrin in lipid cholesterol-enriched rafts. Silencing Sig1R with siRNA chased β1 integrin from lipid rafts, reducing cell adhesion to matrix component such as fibronectin and vitronectin. Interestingly, treatment with the Sig1R agonist SKF10,047 also reduced β1 integrin density within lipid rafts and cell adhesion, an effect that was mimicked by the depletion of membrane cholesterol by methyl-β-cyclodextrin (Palmer et al., 2007).

From these data, it is clear that Sig1R participates on several facets of cancer cell biology. Recently, mutations in Sig1R have been found to cause a form of ALS and frontotemporal lobar degeneration (Luty et al., 2010; Al-Saif et al., 2011; Prause et al., 2013). Whether mutations in Sig1R also occur in cancer tissues is a question that remains to be explored. So far, the common mechanism by which Sig1R or sigma ligands drives cancer cell behavior is not clear. An exciting hypothesis arises from converging studies describing Sig1R as a sterol-dependent, stress-activated chaperone controlling lipid raft formation in the ER and the plasma membrane (PM) [extensively reviewed in Tsai et al. (2009), Hayashi and Su (2010)]. In response to environmental conditions encountered in cancer tissue (hypoxia, nutrient and growth factor deprivation) Sig1R may dynamically trigger various adaptation mechanisms, the nature of which being tightly dependent on the client protein available in a given tumor cell type. At this stage, it is noteworthy that ion channels emerge from the literature as the main client protein family for Sig1R (Hayashi and Su, 2007; Crottes et al., 2011; Balasuriya et al., 2012; Kourrich et al., 2012, 2013).

SIG1R: A MODULATOR OF ION CHANNELS

VOLTAGE-GATED ION CHANNELS

Voltage-gated ion channels (VGIC) are mainly involved in the initiation and shaping of action potentials and global cell excitability (Hodgkin and Huxley, 1952; Hille, 1984). The progress made during the past decade in characterizing the electrical signature of cancer cell has intriguingly extended the initial function of VGIC far beyond the field of exciting cells. Indeed, VGIC are involved in a number of tumor cell processes including mitosis (Weber et al., 2006; Becchetti, 2011), migration (Gillet et al., 2009; Becchetti and Arcangeli, 2010), apoptosis (Lang et al., 2004), adhesion to ECM (Pillozzi and Arcangeli, 2010), angiogenesis (Pillozzi et al., 2007), homing and drug resistance (Pillozzi and Arcangeli, 2010). Interestingly, Sig1R has been shown to interact with K^+, Ca^{2+}, Cl^- and Na^+ channels (Renaudo et al., 2004; Kourrich et al., 2012). Very recent studies have provided some clues about these interactions.

1- Voltage-gated K+ channels (VGKC)

Numerous studies have reported the inhibition of VGKC by sigma ligands in a wide range of cell types (Kennedy and Henderson, 1990; Soriani et al., 1998, 1999a,b; Lupardus et al., 2000; Kourrich et al., 2012). In particular, sigma ligands decrease current density and provoke a leftward shift in the voltage-dependency inactivation (Zera et al., 1996; Soriani et al., 1999a; Aydar et al., 2002). In a study performed in frogs'pituitary cells, it was nonetheless shown that sigma ligands depress the M-current by a rightward shift of the activation curve (Soriani et al., 1999b). The mechanism by which sigma ligands modulate Kv channels has been proposed to be either direct or indirect, depending on the model used. Inside-out patch clamp experiments suggested a direct effect of sigma ligands on Kv channels in rodent neurohypophysal terminals and in small cell lung carcinoma (Wilke et al., 1999; Lupardus et al., 2000). However, in frogs'pituitary cells, the inhibitory effects of the selective Sig1R ligand (+) pentazocine, on both delayed-rectifier and I_A currents, were abolished in the presence of cholera toxin, GTPγS or GDPβS suggesting the involvement of a Gs-protein dependent pathway (Soriani et al., 1998, 1999a). How could these two sets of observation be interpreted? In a recent study, Mei et al. showed that the sigma ligand Cyproheptadine stimulates the Kv2.1-dependent current in cortical neurons in a Sig1R and $G_{i/o}$-dependent manner. The study indeed describes a functional interaction between Sig1R, Kv2.1 and a G-protein coupled receptor (GPCR): the mu-opoid receptor (He et al., 2012). It can then been proposed that sigma ligands, either alter directly the Sig1R/VGKC coupling or modulate functional complexes that integrate Sig1R, VGKC and GPCR. This last hypothesis is strengthened by recent reports demonstrating that Sig1R modulates several GPCR in the brain (i.e. opioid and muscarinic acetyl choline receptors) (Kim et al., 2010), and forms a complex with D1 and D2 dopamine receptors (Navarro et al., 2010, 2013).

Whether Sig1R requires an endogenous/exogenous sigma ligand to modulate VGKC is a crucial question that has been addressed in a few but important reports focusing on the molecular interaction between Sig1R and its partners. In *Xenopus oocytes*, it has been shown that the co-expression of Sig1R with Kv1.4 or Kv1.3 accelerates the inactivation kinetic parameters (Aydar et al., 2002; Kinoshita et al., 2012). In human leukaemic cells, our group found that the silencing of Sig1R by shRNA reduces the endogenous human ether-à-gogo-related gene (hERG; Kv11.1) current density without altering channel voltage dependency or kinetic parameters. Delving into the molecular mechanisms, we observed that the silencing of Sig1R decreases hERG maturation efficiency and diminishes the α-subunit channel stability at the plasma membrane, in turn reducing the number of ion channels available (Crottes et al., 2011). Inasmuch Sig1R co-immunoprecipitates with hERG, these observations are consistent with the idea of a Sig1R protein behaving either like a chaperone or a channel regulatory β-subunit through a protein/protein interaction (Aydar et al., 2002; Crottes et al., 2011; Kinoshita et al., 2012). This hypothesis was further strengthened by a recent report showing that cocaine exposure induces in nucleus accumbens a persistent protein/protein association between Sig-1Rs and Kv1.2 channels. This phenomenon is associated with a redistribution of both proteins from the intracellular compartments to the plasma membrane (Kourrich et al., 2013).

The presence of VGKC is linked to cell proliferation in various cancer types, through the regulation of both the resting (controlling the Ca^{2+} driving force) and cell volume, both phenomenon participating in the cell cycle checkpoints (for review; Wulff et al., 2009; Becchetti, 2011; Felipe et al., 2012). The connection between VGKC, Sig1R and cancer cell proliferation has been first addressed by Renaudo et al. (2004). We observed that selective Sig1R ligands provoke a cell cycle arrest in small cell lung carcinoma and T-ALL cells by blocking the delayed-rectifier and Kv1.3 channels, respectively. In both cases, Sig1R-dependent inhibition of potassium currents resulted in an accumulation of the cyclin inhibitor $p27^{Kip1}$ and a reduction in cyclin A contents, leading to an arrest at the end of the G_1 phase of the cell cycle (Renaudo et al., 2004).

VGKC of the ether-à-gogo family, i.e., hERG and EAG channels, represent a source of highly promising therapeutic targets for cancer. hERG is mainly expressed in the heart, the central nervous system and the endocrine system were it regulates the frequency of action potentials (for review: Vandenberg et al., 2012). In a series of excellent papers, Arcangeli's group has demonstrated that hERG is a tumor marker of myeloid and lymphoid leukaemias, colon and breast carcinoma, ovarian cancer and glioblastoma (Pillozzi et al., 2007, 2011; Pillozzi and Arcangeli, 2010; Arcangeli, 2011). Importantly, they demonstrated that upon β1 integrin stimulation, hERG forms signaling macro-complexes with β1-integrin, the VEGF receptor Flt-1 or the cytokine receptor CXCR4 in lipid rafts. The channel in turn participates in a crosstalk between cancer cells and their microenvironment to promote invasive processes such as motility, angiogenesis, homing and chemoresistance (Pillozzi et al., 2007; Pillozzi and Arcangeli, 2010). As stated above, we recently showed that Sig1R expression stimulates hERG maturation and membrane stability in the chronic myeloid leukaemia cell line K562. Inasmuch Sig1R co-immunoprecipitates with both immature and mature forms of the channel α-subunits, it is suggested that Sig1R not only associates with hERG in the ER, but also drives it to the plasma membrane (Crottes et al., 2011). The question of whether Sig1R is involved in the formation of such hERG-dependent signaling complexes with β-integrins and other partners is an interesting one but has not been addressed yet. This hypothesis deserves further consideration *in vitro* and *in vivo* knowing that both Sig1R silencing and treatment with the sigma ligand igmesine decrease K562 cell adhesion capacity to fibronectin in a hERG-dependent manner (Crottes et al., 2011).

Channels of the EAG family are present in a number of tumor types. Stuhmer's group nicely demonstrated that CHO cells transfected with EAG exhibit a cancerous invasive phenotype *in vitro* and *in vivo* (Hemmerlein et al., 2006; Gomez-Varela et al., 2007; Pardo and Suhmer, 2008). Further signaling studies revealed that EAG-1 (Kv10.1) enhances cell resistance to hypoxia by increasing HIF-1 levels, thus stimulating VEGF secretion (Downie et al., 2008). The over expression of EAG-2 has been recently shown in human medulloblastoma (MB). In this cancer, EAG-2 promotes the progression of the MB tumor by regulating cell volume dynamics, in turn inhibiting the tumor suppressor p38 MAPK pathway (Huang et al., 2012). While the putative link between EAG channels and Sig1R has not been addressed so far, it is tempting to speculate such an interaction considering the molecular and structural proximity between EAG and hERG channels.

2-Voltage-gated Na^+ channels (VGNC)

The existence of VGNC has been first speculated by Hodgkin and Huxley to account for the fast depolarizing phase of the action potential of excitable cells (Hodgkin and Huxley, 1952). To date, the family of VGNC includes nine members mainly involved in the encoding of neuronal signaling, cardiac rhythm, muscle contraction and endocrine secretion (Hille, 1984; Harmar et al., 2009). Intriguingly, VGNC are expressed in metastatic cells of many cancers. In these cells, the sodium current driven by VGNC α subunits enhances the invasion and metastasis *in vivo* (Brisson et al., 2011, 2012; Yang et al., 2012). Expression of the cardiac Nav1.5 α subunit (SCN5A) is correlated with a poor prognosis in breast cancer specimens, suggesting that VGSCs may be used as prognosis marker in cancer progression (House et al., 2010; Yang et al., 2012). The mechanical link between Nav1.5 and cancer progression has been recently documented: in breast cancer cells, Nav1.5 associates with the Na^+/H^+ exchanger NHE1 in caveolae; Nav1.5 stimulates NHE1 activity, contributing to the acidification of the pericellular space. The low extracellular pH in turn potentiates the activity of different cathepsins involved in ECM degradation, a fundamental step for cancer cell invasion process (Gillet et al., 2009; Brisson et al., 2011, 2012). In recent studies, Jackson and Ruoho's groups have shown that sigma ligands reduce Nav1.5-dependent currents in cardiomyocytes of wild type mice. Interestingly, Nav1.5 current sensitivity to sigma ligands was lost in cardiomyocytes of knock-out mice for Sig1R (Fontanilla et al., 2009; Johannessen et al., 2011). The nature of the interaction occurring between Sig1R and Nav1.5 has been scrutinized in 2012 by atomic force microscopy (AFM) which revealed that Sig1R directly binds the channel with a four-fold symmetry in human embryonic kidney cell (HEK) heterologous expression system (Balasuriya et al., 2012). Because the Nav1.5 channel includes the four pore-forming α subunits within a single protein, this result suggests that Sig1R neither interacts with C- nor N-terminus, but rather with the transmembrane domains. This hypothesis is strengthened by the fact that deletions in the transmembrane domain of Kv1.3 subunits abolish their co-immunoprecipitation with Sig1R in *Xenopus* oocytes (Kinoshita et al., 2012). While the suppression of Sig1R expression in mice cardiomyocytes fails to alter any parameter of the native Nav1.5 current (Fontanilla et al., 2009), the Sig1R silencing in the highly aggressive MDA-MB-231 breast cancer cell line results in a strong reduction in current density, suggesting that Sig1R controls Nav1.5 trafficking in cancer cells but not in healthy cardiac cells (Balasuriya et al., 2012). From these observations, it can be hypothesized that Sig1R, by enhancing Nav1.5 membrane expression in breast cancer cells, modulates NHE1 activity, resulting in greater aggressiveness potency.

3-Voltage-gated calcium channels (VGCC)

VGCC are principally involved in fast synaptic transmission, cardiomyocyte and striated muscle contraction, as well as

stimulus-secretion coupling. The low threshold T-type channel (Cav-3) has however been involved in proliferation and differentiation in several cancer cell lines (Gackiere et al., 2008; Prevarskaya et al., 2010; Becchetti, 2011). While no data support an interaction between Sig1R and the T-type channel in the literature, it has been shown that Sig1R co-immunoprecipitate with high-threshold L-type channels in retinal ganglion and that the sigma ligand SKF 10.047 inhibits the corresponding current (Tchedre et al., 2008). These observations suggest that VGCC in cancer cells might be a client for Sig1R.

CALCIUM-ACTIVATED POTASSIUM CHANNELS (KCa)

KCa channels are involved in many physiological processes by regulating calcium entry through the control of the membranes'resting potential and Ca^{2+} driving force. A link between Sig1R and small-conductance KCa channels has been recently proposed in synaptic activity and plasticity in the hippocampus (Martina et al., 2007). In this report, the authors showed that Sig1R ligands potentiate the N-Methyl-D-Aspartate (NMDA) receptor responses and long-term potentiation (LTP) by inhibiting a small conductance Ca^{2+}-activated K^+ current (SK channel). Interestingly, SK3 channels play a predominant role in melanoma and breast cancer cell migration and are considered as potent targets for cancer therapy (Potier et al., 2006; Chantome et al., 2009; Girault et al., 2012). On the other hand, SK4 channels have been involved in the migration potency of glioblastoma stem cells (Ruggieri et al., 2012). The putative interaction between SK channels and Sig1R thus constitutes an interesting hypothesis that remains to be explored.

VOLUME-REGULATED CHLORIDE CHANNELS (VRCC)

VRCC, functionally coupled with K^+ channels, drive cell volume regulation by controlling chloride salt-associated water efflux (Hoffmann et al., 2009). Cell volume regulation and regulatory volume decrease (RVD) participate to at least three main aspects of cancer progression, i.e., cell cycle (G1/S and G2/M volume checkpoints) (Lang et al., 2000; Rouzaire-Dubois et al., 2000; Becchetti, 2011; Hoffmann, 2011; Huang et al., 2012), motility (control of cell shape dynamics through salt and water fluxes) (Cuddapah and Sontheimer, 2011) and apoptosis (Apoptosis Volume Decrease occurring at an early signaling step of programmed cell death) (Bortner and Cidlowski, 2011; Bortner et al., 2012). Our group has shown that sigma ligands strongly inhibit both VRCC and VGKC in T leukaemic and small cell lung carcinoma cells in a Sig1R-dependent manner. VRCC and VGKC inhibition lead to a strong reduction in RVD potency after a hypotonic shock. In isotonic conditions, cell treatment with sigma ligands lead to cell swelling, underlying an arrest of cell division at the late G_1 phase. These results indicate that the pharmacological alteration of Sig1R, by inhibiting channels involved in RVD, can block the cell division process (Renaudo et al., 2004, 2007). In these studies we also questioned the function of Sig1R in cancer cells in the absence of exogenous ligands. We observed that the over-expression of Sig1R in HEK cells was sufficient *per se* to significantly reduce the activation kinetics of VRCC upon hypotonic shock. We proposed that the presence of Sig1R induces a tonic reduction of VRCC activity, not sufficient to impede the cell cycle, but strong enough to protect cells from apoptosis by delaying AVD. This result was confirmed by showing that cells over-expressing Sig1R are less sensitive to staurospaurine-induced apoptosis than normal cells (Renaudo et al., 2007). Together with other reports, this study has unveiled the function of Sig1R as a protein involved in cell protection against environmental stress by modulating ion channels (Hayashi and Su, 2007; Renaudo et al., 2007).

CALCIUM SIGNALING AND ION CHANNELS AT THE MAM

Cell fate largely depends on calcium exchanges occurring between ER and mitochondria. These exchanges generally take place at specific membrane localization, the MAMs, which were originally described as sites for lipid synthesis and lipid transfer between ER and mitochondria membranes (Rusinol et al., 1994) for review: (Parys et al., 2012). Calcium fluxes between the two compartments involve various chaperones and signaling proteins as well as ion channels and transporters including IP3 receptors, voltage-dependent anion channels (VDAC) or the translocon. The regulation of calcium entry in the mitochondria participates in the control of the energy state and cell response to ER-mediated stress. Calcium homeostasis at MAM therefore constitutes a crossroad decision for cell engagement toward apoptosis, survival or autophagy (Tsai et al., 2009; Parys et al., 2012; Hammadi et al., 2013). Not surprisingly, MAM-associated transport machinery is disregulated in the context of environmental challenges in cancer such as hypoxia, low pH and dramatic nutrient deprivation (Moenner et al., 2007; Raturi and Simmen, 2013). As stated above, Hayashi and Su have demonstrated in CHO cells that the Sig1R chaperone plays a fundamental role in regulating the Ca^{2+} transport machinery within the MAMs, leading to a reinforced cell survival in response to environmental stress (Hayashi and Su, 2007). In particular, stress-activated Sig1R chaperones IP3 receptor and prevents its degradation. Moreover a recent report has shown that Sig1R is physically associated to VDAC2, a mitochondrial channel involved in cholesterol import into the mitochodria for metabolic regulation (Marriott et al., 2012). While no data is available on the function of MAM-associated Sig1R in tumors, it is conceivable that Sig1R contributes to the adaptation of cancer cells in restrictive environment.

CONCLUSION AND PERSPECTIVES

In summary, Sig1R is a stress-activated chaperone which controls, through different mechanisms, several families of ion channels at the plasma membrane and at the MAM. Studies realized in the retina, brain and heart strongly suggest that Sig1R participates in cell resistance to tissue injury, for instance infarction, stroke or ischemia (Kourrich et al., 2012). Several reports indicate that Sig1R exerts a role only in conditions of stress and remains generally "silent" in healthy organs or in steady-state conditions (Maurice and Su, 2009; Tsai et al., 2009). In good agreement with this idea, Sig1R KO mice present a normal development and behavior but are less resistant to experimental stroke (Ruscher et al., 2011). Moreover, the absence of side effects of Sig1R ligands in clinical trials in human suffering psychiatric disorders, improves the hypothesis of a dynamic and protective role of Sig1R in stressing conditions (Volz and Stoll, 2004; Banister

and Kassiou, 2012). Thus, it is tempting to speculate that tumor cells hijack the primary protective function of Sig1R to enhance their survival/growing/invasive potency in restrictive metabolic conditions encountered within the tumor tissue. As demonstrated by many authors, the aberrant expression of ion channels confers selective advantages for cancer cells to adapt their behavior and survival in the tumor environment. While research studies mainly focus on the function of one ion channel in a cancer type, it is important to consider that many ion channels are deregulated in the same cancer cell. Because a variety of ion channels are client proteins for Sig1R, we speculate that the Sig1R chaperone controls cancer cells'electrical plasticity by putatively "driving" ion channels to potentiate their function in proliferation, apoptosis resistance, migration and angiogenesis (**Figure 2**). At the time being, there is no real explanation on the process that controls the expression of all these ion channels in cancer cells and it is often postulated that this is due to the acquisition of an embryonic or developmental phenotype. The possibility that Sig1R expression might participate to this phenotype is an interesting hypothesis that has not been explored so far.

The literature strongly argues for a close interaction between Sig1R and ion channels that are already expressed in the cell. An alternative mechanism should however be considered: because

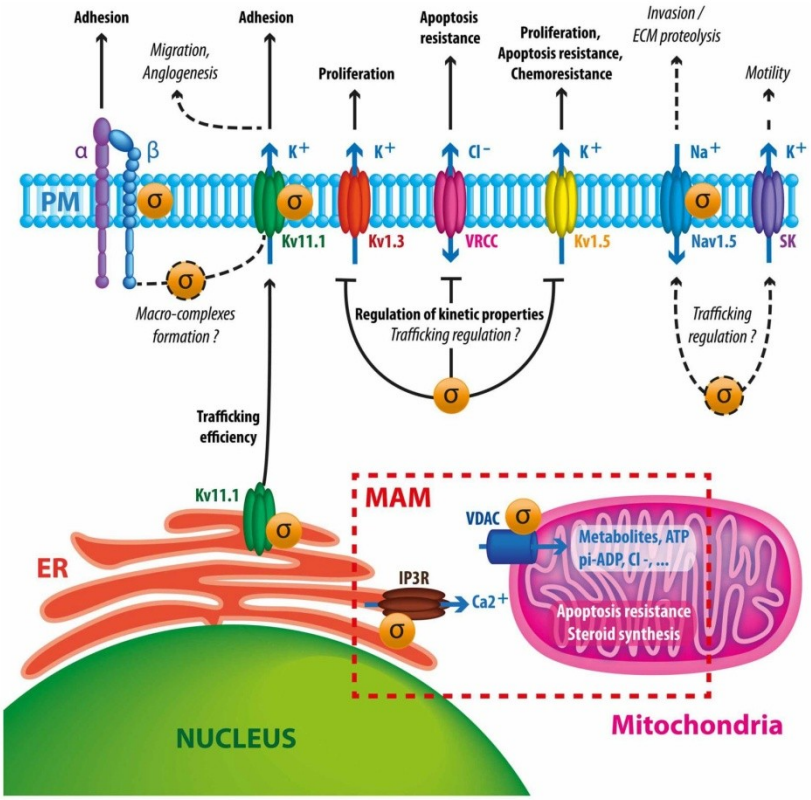

FIGURE 2 | Schematic diagram summarizing putative and documented interactions between Sig1R and ion channels in cancer cells. Sig1R promotes hERG maturation and membrane stability which may potentiate channel interaction with β1 integrin macrocomplexes, leading to enhanced migration and angiogenesis. Sig1R also directly binds to other VGIC such as Kv1.3, Kv1.5, and Nav1.5 controlling either current density or kinetic properties of channels. These interactions may have consequences on cell proliferation, apoptosis resistance, chemoresistance and invasive properties. The presence of Sig1R in cancer cells tonically reduces VRCC activity via a yet unknown mechanism, leading to a better resistance to apoptotic signals. Sigma receptors have also been associated to SK channels, suggesting that they may participate to cancer cell motility. At the MAM, Sig1R may enhance cell survival by regulating calcium fluxes between ER and mitochondria by chaperoning IP3 receptors and VDAC channels.

of the spatial dynamics of Sig1R within the cell, the protein could also behave as a transcriptional factor controlling either directly or indirectly a kit of genes encoding ion channels. While no data supports the presence of Sig1R in the nucleus, many reports have shown the involvement of Sig1R in a number of signaling pathways potentially targeting transcriptional activity (i.e., MAP kinases, PKA, PI3K/AKT, NFk-B, c-Fos, CREB) (for review: Hayashi et al., 2011).

It is noteworthy that ion channels expressed in cancer cells play important functions in healthy organs as well such as in the heart and brain. As a consequence, therapies based on toxins and drugs directly targeting ion channels present major drawbacks for cancer treatment. The unique properties of Sig1R may pave a new avenue to alter ion channels specifically within tumors. In this regard, many outstanding questions need to be addressed to unravel the importance of Sig1R in cancer such as the consequences of Sig1R silencing on the electrical signature of cancer cells and subsequent alteration of their behavior *in vitro* and *in vivo*. Promising anti-tumoral effects have been obtained *in vivo* with exogenous sigma ligands, but the innate function of Sig1R in cancer remains undetermined. Moreover, the molecular mechanisms of Sig1R ligands on Sig1R/ion channel complexes remain to be addressed.

Answers to these questions will open new strategies based on the targeting of Sig1R to target ion channels and associated cancer progression.

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Annexe 3 : The Orai-1 and STIM-1 Complex Controls Human Dendritic Cell Maturation

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The Orai-1 and STIM-1 Complex Controls Human Dendritic Cell Maturation

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Abstract

Ca^{2+} signaling plays an important role in the function of dendritic cells (DC), the professional antigen presenting cells. Here, we described the role of Calcium released activated (CRAC) channels in the maturation and cytokine secretion of human DC. Recent works identified STIM1 and Orai1 in human T lymphocytes as essential for CRAC channel activation. We investigated Ca^{2+} signaling in human DC maturation by imaging intracellular calcium signaling and pharmalogical inhibitors. The DC response to inflammatory mediators or PAMPs (Pathogen-associated molecular patterns) is due to a depletion of intracellular Ca^{2+} stores that results in a store-operated Ca^{2+} entry (SOCE). This Ca^{2+} influx was inhibited by 2-APB and exhibited a Ca^{2+}permeability similar to the CRAC (Calcium-Released Activated Calcium), found in T lymphocytes. Depending on the PAMPs used, SOCE profiles and amplitudes appeared different, suggesting the involvement of different CRAC channels. Using siRNAi, we identified the STIM1 and Orai1 protein complex as one of the main pathways for Ca^{2+} entry for LPS- and TNF-α-induced maturation in DC. Cytokine secretions also seemed to be SOCE-dependent with profile differences depending on the maturating agents since IL-12 and IL10 secretions appeared highly sensitive to 2-APB whereas IFN-γ was less affected. Altogether, these results clearly demonstrate that human DC maturation and cytokine secretions depend on SOCE signaling involving STIM1 and Orai1 proteins.

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Introduction

Dendritic cells are highly specialized antigen presenting cells (APC) able to induce both specific immunity and immune tolerance. Because of these properties, they represent the most important regulating cells of the immune system [1,2]. On the basis of information gathered from the tissue where they reside, DC adjust their functional activity to ensure that protective immunity is favored while unwanted or exaggerated immune responses are prevented. Immature DC become mature DC with functional competences in response to a number of stimuli through numerous cell surface receptors such as cytokine receptors, TLR (Toll-like Receptors) or type C lectins [3,4]. It results in a sustained increase of intracellular Ca^{2+} acting as a second mediator for T and B cell receptor signaling leading to gene activation, cellular proliferation and cytokine secretion [5,6]. The process of DC maturation induced by extracellular ligands plays a key role in the immune responses but the calcium signaling mechanisms involved in this phenomenon are not well understood. A previous study suggested that a Ca^{2+} influx (also named CCE, SOCE or CRAC) might be involved in murine DC maturation [7]. Among different modalities of Ca^{2+} entry, the implication of an ion channel (selective to Ca^{2+}) sensitive to endoplasmic reticulum (ER) Ca^{2+} stocks, named SOC was shown to control this influx. SOCs can be activated by ER Ca^{2+} depletion leading to a Ca^{2+} influx response named SOCE (Store-Operated Calcium Entry). This SOCE allows reloading of ER Ca^{2+} stocks and is involved in the regulation of several physiological functions [8].

Although their presence and their functionality were investigated in murine DC, there are very few data regarding the role of Ca^{2+} homeostasis and the role of ion channels in human DC. The use of a calcium ionophore induced a mature phenotype in human DC [9], as well as an inhibition of IL-12 secretion [10]. Bagley et al. showed the involvement of PLC (Phospholipase C) in the maturation process of DC induced by LPS [11]. Indeed, PLC activation induced Inositol Triphosphate (IP_3) synthesis, which in turn induced ER Ca^{2+} stocks release through activation of IP_3 Receptor (IP_3R).

Store-operated Ca^{2+} entry (SOCE) appears to be the main mechanism used by many cell types to initiate signal transduction [12]. We choose to focus on two recently discovered molecules: Orai1 and STIM-1 [13]. These two proteins, as they move close to MHC complex in the immunological synapse, are up-regulated during T cell activation and provoke cell polarization [14,15]. One of the implications of Orai1's and STIM-1's up-regulation in the early stages of the immune response is to amplify and ensure

CRAC channel-mediated Ca^{2+} signaling for clonal expansion, differentiation and calcium-dependent regulation of gene expression in T cells. Severe combined immunodeficiency (SCID) patients have been associated with lack of both functional ORAI and STIM-1 [16,17]. Although their presence and their functionality were investigated in mouse DC, there are very few data regarding the role of these proteins in Ca^{2+} homeostasis and the role of ion channels in human DC.

These data clearly indicate that Ca^{2+} signaling via the Orai1/STIM1 complex is likely to be of importance in immunological regulation. We aim to investigate the mechanisms by which Ca^{2+} participates in DC maturation. To do so we analyzed early events of DC maturation process and functional consequences of its inhibition.

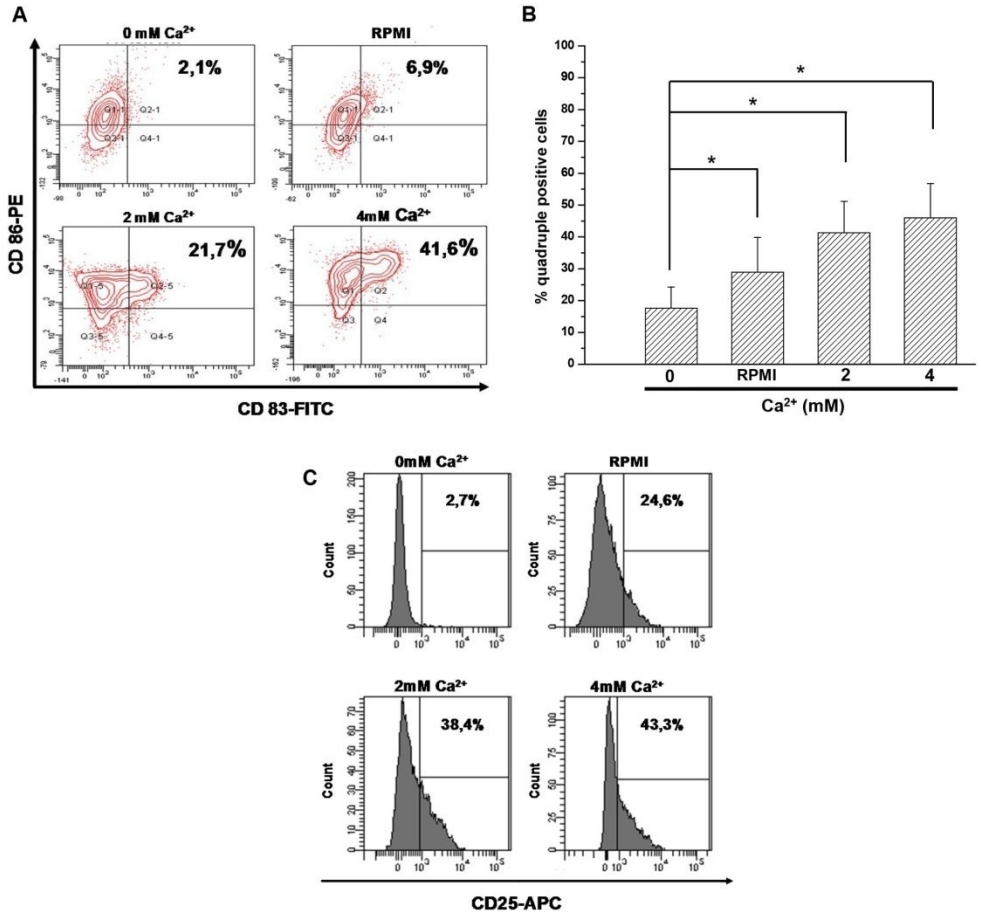

Figure 1. Expression of maturation markers in human dendritic cells according to extracellular Ca^{2+} concentration. DC were cultured with increasing concentrations of Ca^{2+}**(in mM)**. Cells were harvested and double or quadruple staining was assessed by FACS analysis for the 4 conditions. **A** – *CD83 expression is increased by extracellular Ca2+ concentration*. The population showed on this panel is gated on DC-Sign labeling. The percentage of double (CD86 and CD83) positive cells is shown in panel A. Results are representative of 7 independent experiments. **B** – *Expression of maturation markers in relation to extracellular Ca^{2+} concentrations*. The same population expressed CD80, CD86, HLA-DR and CD83 in increasing proportions in increasing extracellular Ca^{2+} concentration. The percentage of quadruple positive cells is shown in panel B. Graph bars represent the mean of 6 independent experiments (mean ± SD, *$p<0,05$). **C** – *CD25 expression in relation to extracellular Ca^{2+}*. Grey histograms represent the expression of the cell-surface marker CD25. Results represent one out of 7 independent experiments.
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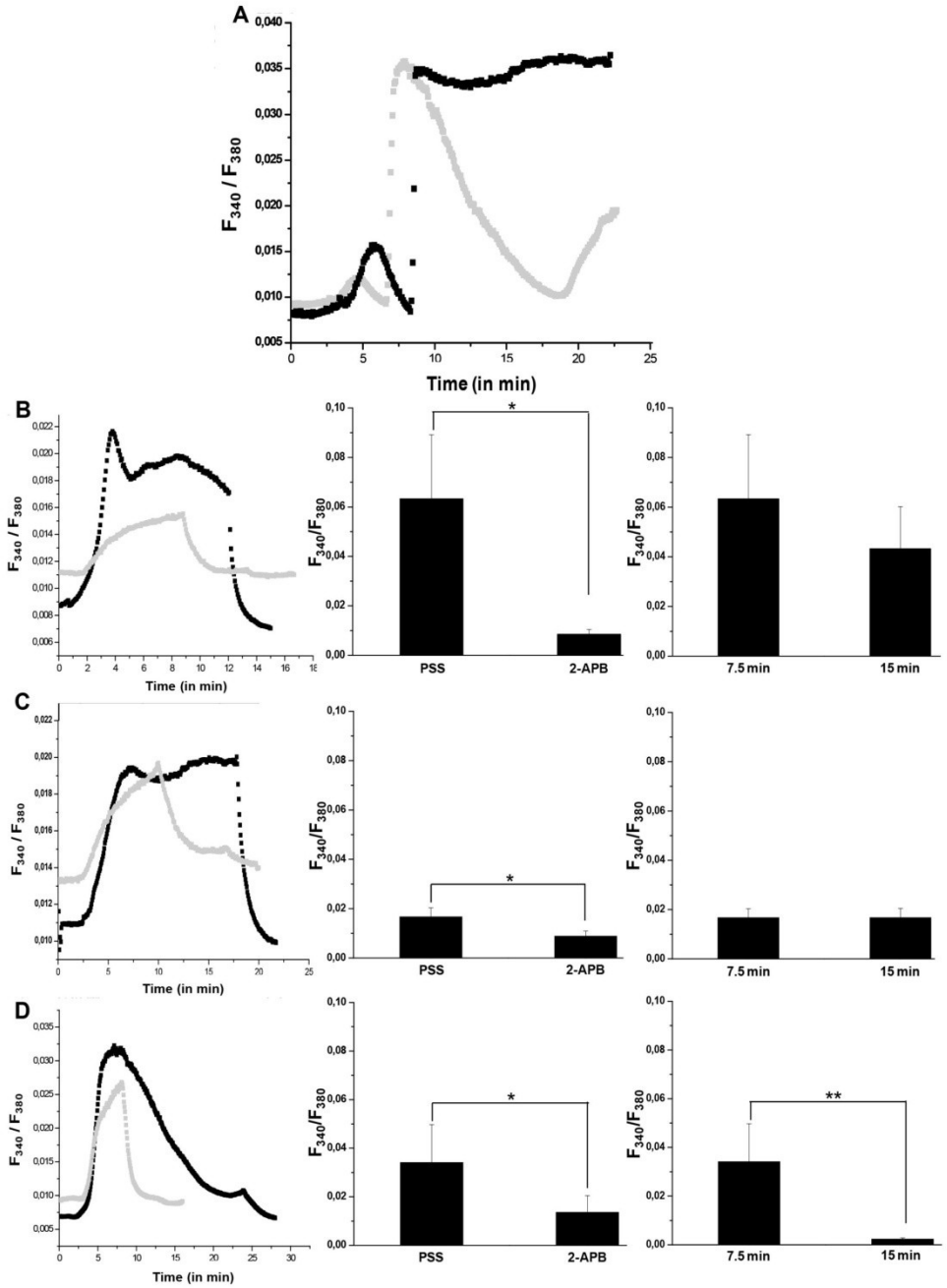

A
F340 / F380
Time (in min)
B
C
D
PSS
2-APB
7.5 min
15 min
*
**

Figure 2. Identification of SOCE during the earlier events of DC maturation. DC were treated with thapsigargin (TG) in calcium free PSS, then PSS supplemented with 2 mM Ca^{2+} was added (**A**). 100 μM 2-APB PSS solution induced a rapid and reversible decrease of $[Ca^{2+}]_i$ (Grey trace). According to different maturation signals (LPS, **B**; TNF-α, **C** or zymosan, **D**), the variations of $[Ca^{2+}]_i$ in the presence of SOCE inhibitor (Grey trace, at 100 μM 2-APB) or its absence (Black trace) were observed by microspectrofluorimetry on left panels. Results are representative of 7 independent experiments. Central panels represent the maximal amplitude mean of CCE according to the treatment. Right panels represent the CCE amplitude mean at 7.5 and 15 min. Means were obtained from 7 independent experiments.
doi:10.1371/journal.pone.0061595.g002

Materials and Methods

1. Generation of Dendritic Cells from Peripheral Blood Mononuclear Cells (PBMC)

Healthy volunteer's blood was obtained by cytapheresis after signed informed consent managed by the French Blood Department. PBMCs were isolated by Ficoll Hypaque density gradient centrifugation (density 1.077, Lymphoprep; Abcys SA, France) and resuspended in culture medium (RPMI, 10% SVF, 1% Penycillin-Streptomycin, 1% L-Glutamine) after 2 washings. Monocyte-derived dendritic cells were prepared after selective adhesion of monocytes to plastic as previously described [18]. Briefly, PBMC were incubated for 45 min at 37°C in 5% CO2 in culture flasks (100 million in a 75 cm^2-Falcon flask, Becton Dickinson, Mountain View, CA, USA), non-adherent cells were discarded and medium containing rhGM-CSF (1000 U/ml) and rhIL-4 (25 ng/ml) was added. Cells were suspended in culture medium (containing 1000 U/ml rhGM-CSF and 25 ng/ml rhIL-4). On day 5, immature DC were harvested (DC-SIGN+/HLA-DR+ >95%), washed and suspended in culture medium with IL-4 and GM-CSF.

2. Cell treatment

DC were cultured during 18 hours in the presence of several concentrations of Ca^{2+} as following; RPMI at 0.4 mM, RPMI with BAPTA 10 mM to obtain Ca^{2+} free solution, RPMI with $CaCl_2$ 2 mM and RPMI with $CaCl_2$ 4 mM. TLR agonists (LPS (50 ng/ml), zymosan (25 μg/ml) and TNF-α (20 ng/ml)) as well as Ca^{2+} inhibitors such as Thapsigargin (750 nM), D609 (100 μM) and 2-APB (100 μM) were added directly to immature DC for 18 hours in individual wells. All drugs and chemicals were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Cells were harvested, washed and used for analyses in flow cytometry, mixed culture reaction or spectrofluorometric analysis. For reagents suspended in dimethyl sulfoxide (DMSO), an equal volume of DMSO was added to control cultures. At the concentrations used, DMSO has no inhibitory or stimulatory effects on DC (data not shown).

3. Solutions

The physiological saline solution (PSS) had the following composition (in mM): NaCl 140, KCL 5.4, $MgCl_2$ 1, NaH_2PO_4 0.33, $CaCl_2$ 1.8, D-Glucose 11.1 and HEPES 10; adjusted to 7.4 with 1 M NaOH. The Ca-free solution had the following composition (in mM): NaCl 140, KCL 5.4, $MgCl_2$ 1, NaH_2PO_4 0.33, EGTA 1, D-Glucose 11.1 and HEPES 10; adjusted to 7.4 with 1 M NaOH. Thapsigargin (TG), 2-ABP (2-AminoPhenyl Borate), LPS (Lipopolysaccharide), zymosan and TNF-α were added to the PSS at concentrations indicated in the figure legends.

4. Fluorescence measurements

Intracellular Ca^{2+} concentrations were estimated using the ratiometric fluorescent dye Fura-2. Immature DC were plated on cover slips (Fluorodish FD35-100, WPI, UK) coated with poly-L-Lysin (Sigma-Aldrich, St Quentin Fallavier, France) in PSS. Cells were incubated in PSS containing Fura-2 AM (5 μM) (Sigma-Aldrich, St Quentin Fallavier, France), the membrane-permeant acetoxymethyl ester form of Fura-2, diluted in pluronic acid-F127 (Sigma-Aldrich, St Quentin Fallavier, France), during 60–75 min at 37°C. Cells were then washed with PSS and left for 2 additional minutes before recording.

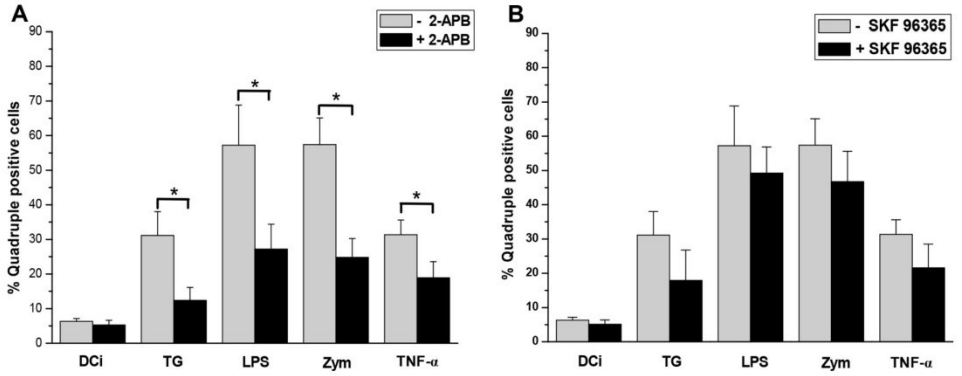


Figure 3. 2-APB decreased the expression of maturation markers in human DC. In panel **A**, DC were cultured in medium with maturating agents (TG (750 nM), LPS (50 ng/ml), Zymosan (25 μg/ml) or TNF-α (20 ng/ml)) in the absence or presence of 2-APB (mean of percentage ± SD, *p<0,05, n = 7). In panel **B**, DC were cultured in medium with the maturating agents in the absence or presence of SKF 96365 (100 μM) (n = 7 for DCi, TG and LPS conditions and n = 4 for Zymosan and TNF-α). The marker expressions were analyzed by FACS as described for figure 1B. In panel C, ORAI-1 and STIM-1 protein expressions were analyzed by western blotting to control Si-RNA efficacy.
doi:10.1371/journal.pone.0061595.g003

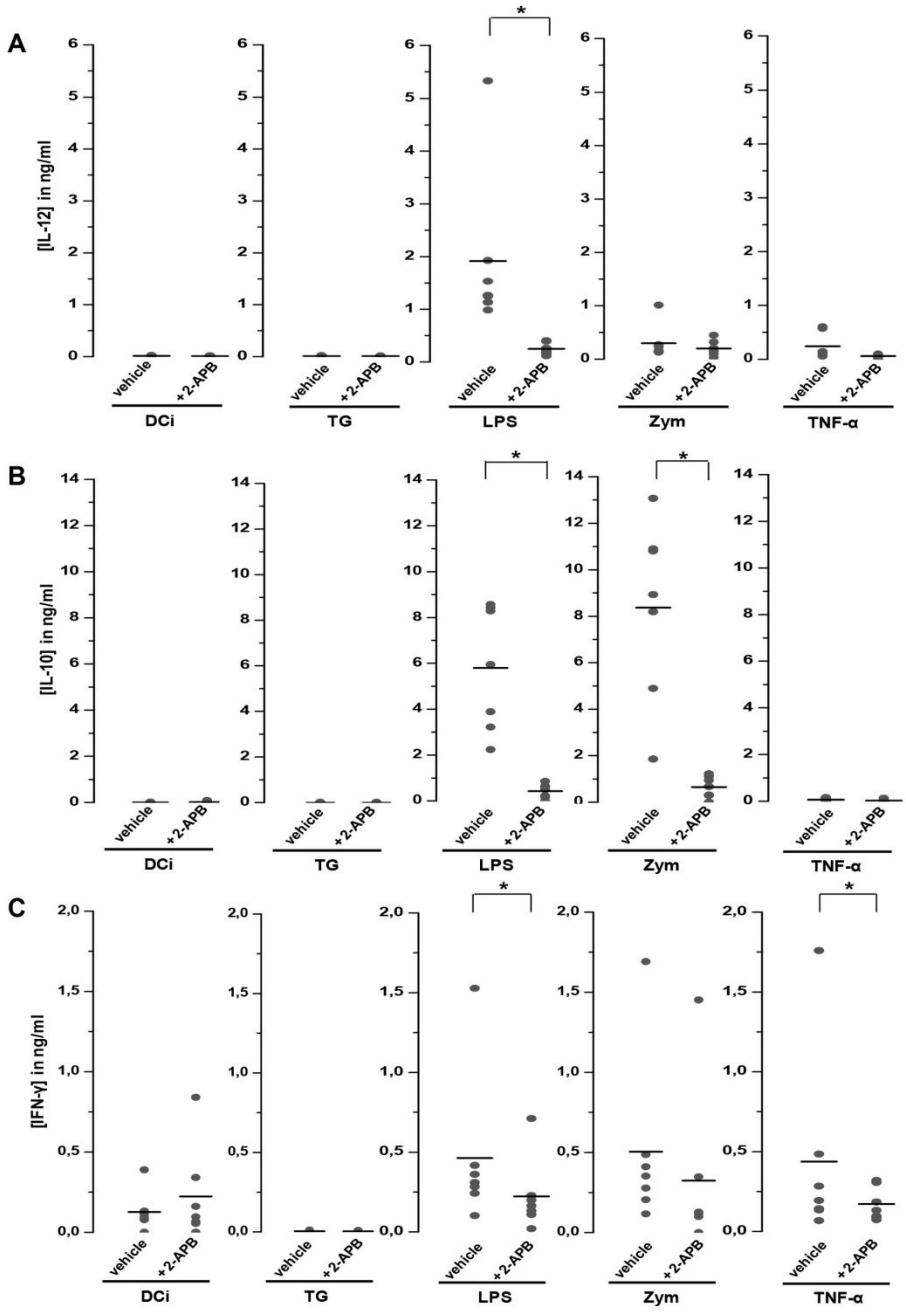

A
[IL-12] in ng/ml
B
[IL-10] in ng/ml
C
[IFN-γ] in ng/ml
vehicle
+2-APB
DCi
TG
LPS
Zym
TNF-α
*

Figure 4. 2-APB decreased the cytokine secretions in human DC. IL-12 (panel **A**), IL-10 (panel **B**) and IFN-γ (panel **C**) were measured in supernatants of DC treated by maturating agents (TG (750 nM), LPS (50 ng/ml), zymosan (25 μg/ml) or TNF-α (20 ng/ml)) for 18 h in the presence of 100 μM 2-APB. In each graph, the black line represents the mean of 7 experiments, (*p<0,05).
doi:10.1371/journal.pone.0061595.g004

Samples were analyzed with a Nikon Eclipse TE2000-S inverted epi-illumination microscope (Nikon, France). The excitation light source was a 75-W Xenon arc lamp. Excitation light at the two-excitation wavelengths maxima of Fura-2 (340/380 nm) was chopped by a monochromator (Cairn Optoscan, UK). The excitation protocol was a 50 ms excitation at each wavelength every 2 s. Excitation light was directed through a 60× oil immersion objective with a numerical aperture of 1.4 (Nikon Plan Apo, France). Fluorescence emissions at 510±20 nm were detected by a photomultiplier tube (PMT) placed on the side of the microscope. The analogical signals of PMT were digitized by a Digidata 1322, a converter (Axon Instrument, USA) at a sampling frequency of 2 kHz. This numeric signal was analyzed using Clampex 8.2 (Axon Instrument, USA). Background fluorescence was determined at 340 and 380 nm from an area of the dish free of cells after experiment and was routinely subtracted.

5. Flow Cytometric Analysis

Dendritic cells were incubated at 4°C with saturating concentrations of fluorochrome-conjugated mAbs (CD80, CD86, CD83, CD25, HLA-DR and DC-SIGN) in the dark for 30 min, washed twice with PBS, fixed in 0.5% PFA-PBS solution and analyzed with a FACSCanto (Becton Dickinson, France). Dead cells were gated out on the basis of their light scatter properties. Data were analyzed with Diva Software.

For intracellular detection of IFN-γ, cells were incubated in Golgistop solution (Becton-Dickinson) for the last 5 hours of culture; next the cells were permeabilized with BD cytofix/cytoperm solution (Becton-Dickinson, France) and finally labeled with an APC-coupled antibody specific for IFN-γ (Becton-Dickinson, France) according to the manufacturer's instructions.

6. Enzyme-linked immunosorbent assays (ELISA)

Cell culture supernatants were harvested and stored at −80°C until assayed for cytokines. Human IL-10, IFN-γ and IL-12 p70 concentrations were measured by ELISA using Ready-Set-Go kits from e-Bioscience (Montrouge, France) according to the manufacturer's instructions. Briefly, microtiter plates were coated overnight at 4°C with antibodies specific for IL-12p70, IL-10 and IFN-γ, from e-Bioscience (Montrouge, France). The plates were washed and blocked according to the manufacturer's instructions. Samples and standards were analyzed in triplicate, and tested with the avidin-peroxydase system. Optical densities were measured in an ELISA plate-reader at 450 nm wavelengths.

7. RNA extraction and Real-time PCR

Total RNA was isolated using RNA extraction minikit (Qiagen, Germany) following the manufacturer's protocol. RNA yield and purity were determined by spectrophotometry, and only samples with an A260/A280 ratio above 1.6 were kept for further experiments. Prior to reverse transcription, total RNA was treated with DNAase I for 30 min at room temperature. RNA was then reverse-transcribed using Superscript II reverse transcriptase and oligodT (Invitrogen, France). Quantitative (real time) PCR experiments were performed with a Lightcycler 480 (Roche, France). The PCR protocol consisted in a denaturizing step at 95°C for 2 min, followed by 35 cycles of amplification at 95°C for 15 s, 60°C for 30 s, and 72°C for 10 s. The experiments were performed in duplicate, and negative controls containing water instead of first strand cDNA were done. The results were calculated with the ΔCt method, where the parameter Ct (threshold cycle) is defined as the fractional cycle number at which the PCR reporter signal passes a fixed threshold. Primers used for PCR experiments had the following sequences (expected sizes): STIM-1, forward 5′-GCATCTTGCCTGGAGACCGT-3′ and reverse 5′-CAAGACGGACGCATACATCC-3′; ORAI1, forward 5′- GTCACCTACCCGGACTGGAT-3′ and reverse 5′- TGGAGGCTTTAAGCTTGGCG-3′. In order to prevent amplification of genomic DNA, forward and reverse primers used spanned neighboring exons.

8. Immunofluorescence and confocal analysis

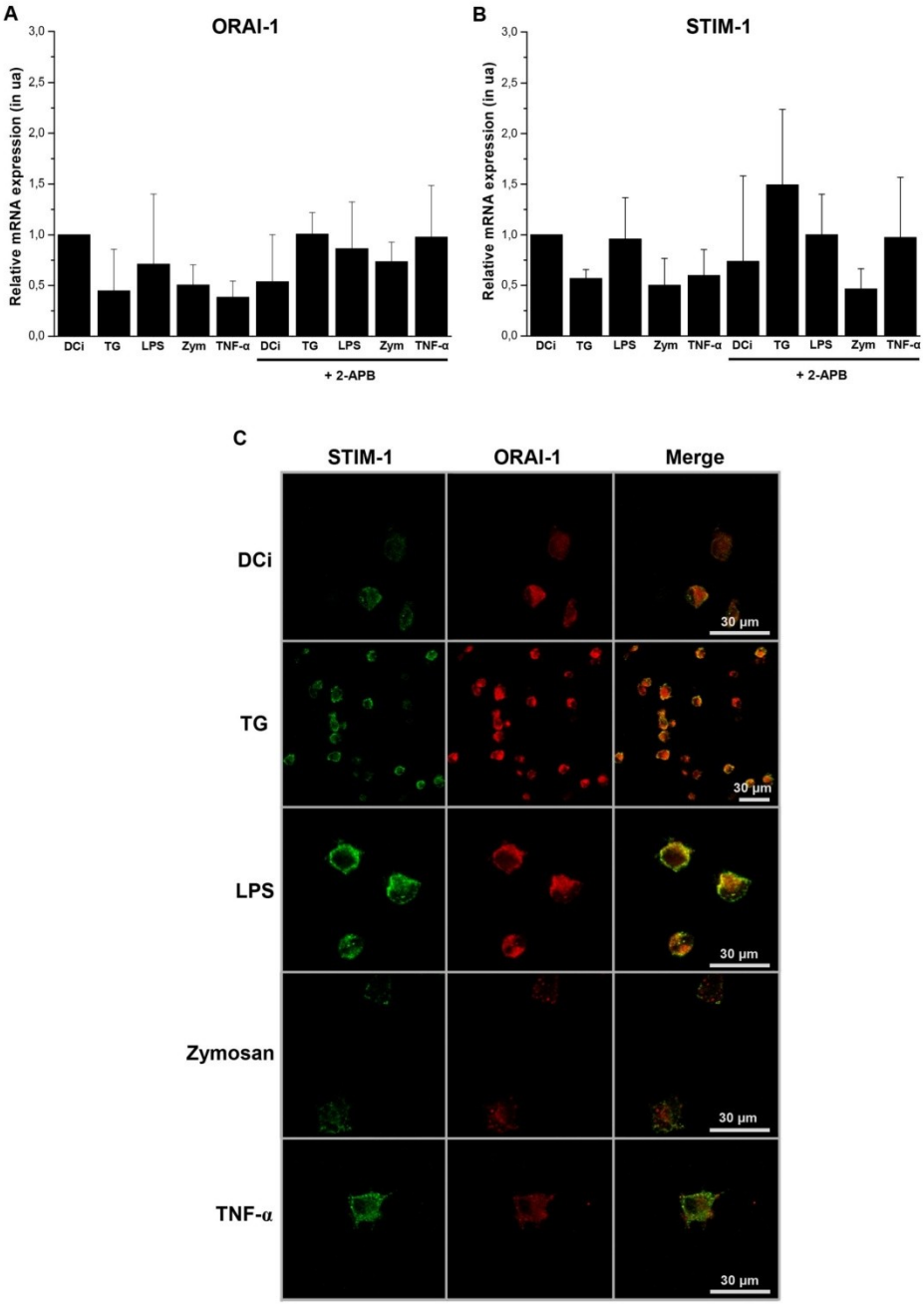
For immunofluorescence labeling of STIM1 and Orai1, DC were spun on glass LAB-TEK (Nunc International, USA) coated with Poly-L Lysine (Sigma-Aldrich, France). Cells were permeabilized and fixed by BD Cytofix/Cytoperm ^{TM} Plus kit (BD Biosciences, USA) in labeling medium (PBS with 4% FBS and 0,1% Azide) at room temperature. After fixation, samples were then incubated with purified antibodies diluted in labeling medium during 1 hour at room temperature. As primary reagent, we used as primary antibody a monoclonal mouse anti-human STIM-1 (Santa Cruz USA) and polyclonal rabbit anti-human Orai1 (Santa Cruz, USA). After washing cells twice with labeling medium, specific staining was detected by Alexa Fluor 488 conjugated goat anti-mouse IgG and Alexa Fluor 564 conjugated goat anti-rabbit IgG (Invitrogen, France), for STIM-1 and Orai1 respectively. Cover slips were placed in mounting medium (Invitrogen, France) and visualized with a LMS510 meta Zeiss confocal microscope equipped with a x63 water immersion objective (NA: 1. 2). Images shown are single optical slides, with the double labeling.

9. RNA interference

Pre-designed si-RNAs (Santa Cruz Biotech, USA) specific for STIM1 or Orai1 were used to inhibit STIM1 and Orai1 expression. In each experiment, 4.10^5 immature DC were plated in 24-well plates in 400 μl growth medium (Opti-MEM containing 10% FBS without antibiotic). Lipofectamine RNAi-Max (1 μl/well; Invitrogen, France) was first diluted in 50 μl Opti-MEM (Invitrogen, France) for 5 minutes before being mixed with an equal volume of Opti-MEM containing 20pmol of siRNA. After 20 minutes, 100 μl of the Lipofectamine/siRNA mixture were added to the cells to perform siRNA transfection. Fresh growth medium (500 μl) was added 4 hours after transfection. Cells were cultured for 36 hours at 37°C to obtain optimal silencing of targeted genes. The efficiency of gene silencing was evaluated by western blot and flow cytometry. A siRNA Control (Santa Cruz Biotech, USA) was tested as negative control. The sequence of this siRNA Control is not specific to any genes. Interference efficiencies were evaluated by western blotting using a monoclonal antibody anti-STIM-1 (clone H180) and polyclonal anti-Orai-1 (clone H46) from Santa Cruz Biotechnology.

10. Statistical analysis

Statistical analyses were performed using a nonparametric Mann – Whitney test with XLSTAT 2007 software. For all tests, a p-value of <0.05 was considered statistically significant.


A
ORAI-1
Relative mRNA expression (in ua)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
DCi
TG
LPS
Zym
TNF-α
DCi
TG
LPS
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+ 2-APB
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STIM-1
Relative mRNA expression (in ua)
3,0
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30 µm
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Figure 5. Localization of STIM-1 and Orai-1 in DC. The expression of Orai-1 and STIM-1 was analyzed at the transcriptional (**A** and **B**) or translational (**C**) level. Quantitative RT-PCR analysis of Orai-1 (**A**) and STIM-1 (**B**) mRNA showed their expression profile for different maturating stimuli (mean ± SD). The protein expressions of STIM-1 and Orai-1 were analyzed by confocal microscopy using specific antibodies coupled with a secondary antibody: Alexa Fluor®-488 for STIM-1 (**green**) and Alexa Fluor®-555 for Orai-1 (**red**). The cells were treated with maturating agents (TG (750 nM), LPS (50 ng/ml), zymosan (25 μg/ml) or TNF-α (20 ng/ml)) during 18 hours.
doi:10.1371/journal.pone.0061595.g005

Results

1. Extracellular calcium induces mature DC phenotype

We evaluated the expression of DC cell surface markers of maturation in presence of different calcium concentrations by flow cytometry. An increased extracellular Ca^{2+} concentration resulted in an increase of expression of CD83 and CD86 (Fig. 1A). We analyzed the percentage of DC quadruple positive cells (CD86/HLA-DR, CD80, CD83) in Fig. 1B. This percentage increased in parallel with the extracellular Ca^{2+} concentration (from 2 to 4 mM). The surface expression of CD25 increased from 24.6% to 43.3% when the extracellular Ca^{2+} concentration increased from 0.4 to 4 mM (Fig. 1C). In comparison, only 2.7% of DC expressed CD25 at their surface in the absence of Ca^{2+}.

2. CRAC channel-mediated Ca^{2+} influx promotes DC maturation

Since we demonstrated that extracellular calcium was able to modify the expression of phenotype markers in DC, we hypothesized that a calcium entry occurs during the earlier events of DC maturation. In Ca^{2+} free solution, TG induces store depletion. Calcium reintroduction induces a CCE sensitive to 2-APB. So, a SOCE following calcium store depletion (from ER stocks) was observed in human DC after application of TG, and it was sensitive to 2-APB (Fig. 2A). In addition, DC stimulation with TLR agonists resulted in a rapid and sustained increase in $[Ca^{2+}]_i$ suggesting that maturation was accompanied by CRAC channels activation (Fig. 2B, 2C and 2D). PSS solution perfusion with 100 μM 2-ABP significantly reduced but did not fully eliminate $[Ca^{2+}]i$ increase following LPS, zymosan or TNF-α treatments.

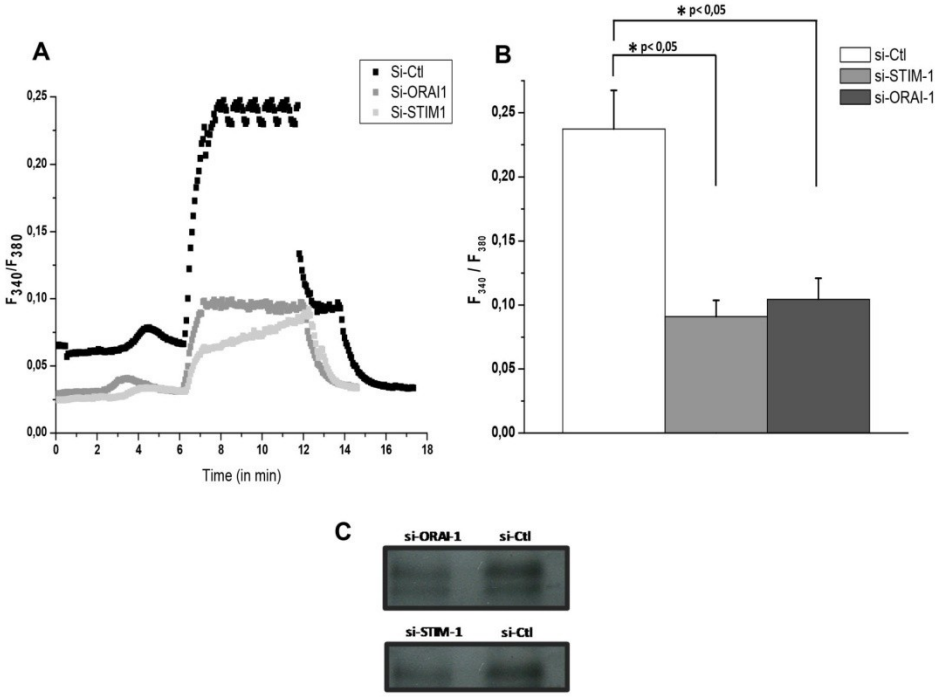

Figure 6. Inhibition of SOCE in DC by si-RNA (si-STIM-1 and si-Orai-1). CCE was analyzed on DC treated by specific Orai-1 (grey line) and STIM-1 (light grey line) si-RNAs for 36 h; DC were exposed to TG (in Ca^{2+}-free solution) next, $Ca^{2+}$was re-introduced (PSS, Ca^{2+} at 2 mM), a PSS solution with 2-APB (100 μM) was perfused on si-Ctl cells (black line) by microspectrofluorimetry (**A**). In panel **B**, the mean amplitude of intracellular Ca^{2+} concentration is represented by bar graphs (5 experiments, mean ± SD, *p<0,05). The siRNA efficiencies were controlled by protein expression analysis using western blotting (**C**).
doi:10.1371/journal.pone.0061595.g006

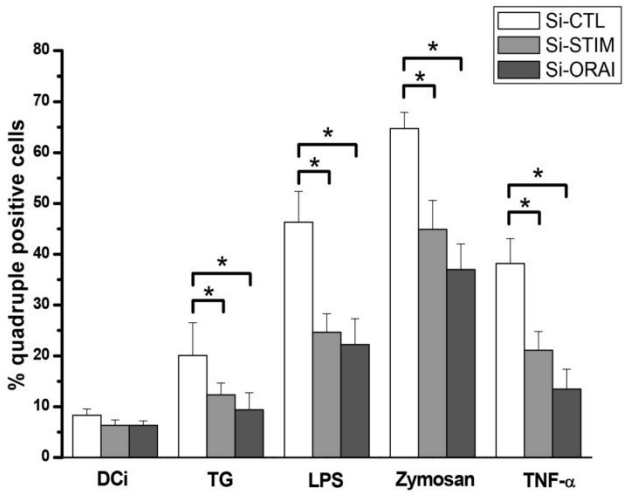

Figure 7. Orai-1 and STIM-1 inhibition decreased the maturation marker expressions on DC. DC treated by si-Ctl (white), by si-STIM-1 (light grey) or by si-Orai-1 (dark grey) were maturated by following maturating agents (TG at 750 nM, LPS at 50 ng/ml, zymosan at 25 µg/ml or TNF-α at 20 ng/ml) for 18 h. Then, cells were harvested and quadruple staining (CD80, CD86, CD83, CD25) was assessed by FACS analysis for each condition. The results represent the mean of percentage of quadruple positive cells from 5 independent experiments (mean ± SD, *p<0,05).
doi:10.1371/journal.pone.0061595.g007

For LPS, Ca^{2+} entry presented higher amplitude compared to those observed with zymosan and TNF-α (0.061 versus 0.034 and 0.018). Moreover, the continuous component in SOCE was sensitive to 2-APB after 10 min (Fig. 2B and 2C). In contrast, only a transient and reversible increase of $[Ca^{2+}]$ was observed in zymosan condition (Fig. 2D). A decrease of the zymosan-induced SOCE amplitude was observed in presence of 2-APB with a lesser effect compared to LPS and TNF-α (0.016 versus 0.008 and 0.009) (Fig. 2D versus 2B and 2C).

3. Both maturation and cytokine secretions depend on CRAC channels in DC

In DCi, quadruple positive cells represented 6.3% of the DC population. During the next 18 hours of culture in presence of different maturating agents, the expression of these markers increased up to 31.1% with TG, 57.2% with LPS, 57.3% with zymosan and 31.4% with TNF-α treatment (Fig. 3) As shown in figure 3A, the treatment of DC by 2-APB during 18 h decreased the percentage of quadruple positive cells ($p<0.005$) to 50% for all conditions. Treatments with SKF 96365 did not significantly change the marker expression level on DC whatever the maturating agents (Fig. 3B).

Cytokine secretion by DC is an essential step leading to T cell activation and polarization. We therefore evaluated DC cytokine secretion by ELISA using different inhibitors (TG or 2-APB) and maturating agents. TG-treated DC were not able to secrete any cytokine tested (Fig. 4). In our conditions, LPS-treated DC secreted IL-12, IL-10 and zymosan-treated DC secreted IL-10 (Fig. 4A and 4B). These cytokine secretions were abolished in presence of 2-APB (Fig. 4) or SKF 96365, another inhibitor of SOCE (data not shown). Secreted IFN-γ was also significantly reduced for TNF-α and LPS -DC ($p<0.05$) by 2-APB addition and not for zymosan-matured DC.

4. Both Orai1 and STIM1 proteins are expressed in DC

In order to show both Orai1 and STIM1 mRNA and proteins on DC, quantitative RT-PCR (Fig. 5A, and 5B) and confocal microscopy (Fig. 5C) analyses were conducted using specific primers and fluorescent-labeled antibodies. The Orai1 transcript was never regulated whatever the treatment conditions (Fig. 5A). The Orai1 transcript was over-expressed in all culture conditions in the presence of 2-APB (Fig. 5A). Comparatively, STIM-1 transcript expressed by DCi and its expression was unmodified by all maturating agents (Fig. 5B). Treatment with 2-APB did not affect STIM-1's expression at the transcriptional level, except in TG condition in which STIM-1 mRNA seems to be up-expressed (Fig. 5B). The Orai2 and Orai3 mRNA expressions were not modified by the different treatments as tested in real-time PCR. By the same way, STIM2 mRNAs were not observed whatever the DC maturation condition (data not shown).

We then evaluated the protein expression of Orai1 and STIM-1 in DC, at 18 h of maturation, by confocal microscopy (Fig. 5C). These two proteins appeared to be co-localized on the same cells for all conditions except for zymosan. In this condition, puncta of Orai1 (in red) were observed and STIM-1 (in green) was not detectable in the structures, as it appeared homogeneously distributed in DC (Fig. 5C).

5. Inhibition of both Orai1 and STIM-1 affects DC functionality

2APB is a non-selective blocker of store-operated channels and even it can block other channels. To determine more accurately the channels involved in the process of maturation of DCs, we chose to evaluate the involvement of Orai1 and STIM1 in DC functionality. To do so, a siRNA strategy was used against these proteins in presence of maturating agents. As we obtained an efficient inhibition (Fig. 6C), we first checked whether a SOCE was

observed in the presence of a siRNA control (Black trace in Fig. 6A). Ca^{2+} (2 mM) was re-introduced in a perfused solution, after a treatment by 750 nM TG in Ca^{2+} free solution. This re-introduction provoked a sustained increase of intracellular calcium in DC (F340/F380 around 0.25). The profile of this SOCE is comparable to that obtained in figure 2A. In Si-STIM1 condition, the SOCE induced by TG treatment decreased (F340/F380 around 0.09). The SOCE amplitude was equally diminished by a treatment with Si-Orai1 (F340/F380 around 0.10) (Fig. 6B).

The involvement of Orai1 and STIM1 in DC maturation was also studied by the analysis of surface markers' expressions such as CD83, CD86, CD80, and HLA-DR. The increase of the expression of these markers observed in figure 3, with TG, LPS, zymosan and TNF-α was reduced by a treatment with si-STIM1 or si-Orai1 (Fig. 7).

Discussion

The data reported here clearly underline the importance of Ca^{2+} in human dendritic cells (DC) maturation, and support an important role for Orai1 and STIM1 in DC functions. Furthermore, we report for the first time that maturating agents, such as TLR agonists or cytokines, act through a SOCE in DC. The stimulation of DC with TLR agonists induces a SOCE (Store Operated Calcium Entry) which is inhibited by 2-APB (100 μM), a known inhibitor for this kind of Ca^{2+} entry. This SOCE is involved in DC functions as demonstrated by the 2-APB inhibition of the expression of maturation markers (principally CD25 and CD83) and of the production of cytokines (IL-12, IL-10 or IFN-γ). These results were confirmed for both properties in DC through si-RNA experiments. So, we identified the STIM-1 and Orai1 channel complex as one of the main calcium pathway for DC maturation.

In mouse DC models, different SOCEs have been shown using TG and nifedipine (a L-type calcium channel inhibitor) [7]. The evidenced SOCE appears following a total Ca^{2+} depletion from the ER. Previous work has shown that CRAC channels are one of the main calcium pathways in the mouse [19]. The ion channels responsible for this depletion are IP_3R or members of the RyR family (RyR1, RyR2 or RyR3). In murine DC, the RyR1 protein was shown to be functional contrary to what has been reported in human DC [20,21]. In our hands, the SOCE profiles so obtained distinguished two types of maturating agents, those such as TNF-α and LPS, able to induce an irreversible SOCE with two components, and zymosan that induced a reversible SOCE with a unique component. The SOCE induced by TNF-α and LPS comprised one component that appeared 2-APB-insensitive and transient, and another, which was sustained and 2-ABP-sensitive. As for zymosan, the SOCE was reversible upon washing and less sensitive to 2-APB. This SOCE reversibility suggested that there are at least two different Ca^{2+} pathways used for SOCE in DC. Moreover, the SOCE amplitude also discriminated two categories of maturating agents, those inducing a SOCE with high amplitude such as LPS and those inducing a lower response such as TNF-α and zymosan. Dantrolene (a RyR1 inhibitor) had no effect on the SOCE induced in DC by TG, LPS, zymosan and TNF-α (data not shown). Our characterization of these SOCEs is mainly based on pharmacological studies using 2-APB, which is known to block different channels such as TRPC 1, 3, 4, 5 and 6, or SKF 96365, a potent inhibitor of TRPC 6 and 7 [22]. The SOCE induced by TG or TLR agonists in DC, was inhibited by SKF 96365 (Fig. 3B), suggesting the involvement of TRPC 6 and 7 as it has been observed in cancer cells [23]. The 2-APB specificity did not allowed the discrimination of the respective role of IP3 receptors and the membrane channels responsible of the CCE observed.

Our most striking finding was that the principal component of the SOCE induced by store-depletion appears mainly due to Orai1 and STIM1, the CRAC channel pore subunit and its activator, respectively, which are rapidly recruited to induce a Ca^{2+} influx after TLR or TNF-R activation. In mast cells, these two proteins are recruited by the activation of FcεR leading to the Ca^{2+} increase necessary to degranulation [24]. For mouse DC maturation, STIM2 seems to be the main component of the SOCE channel in the early maturation events [25]. In human DC, STIM -2 expression was not observed. But, we showed that both STIM-1 and Orai1 mRNA and protein expressions are modulated in DC treated with different maturating agents. The complex Orai1 – STIM1 appeared as mainly involved in the maturation by TNF-α and LPS but to a lower extend by zymosan. A treatment of DC with specific si-RNA (si-STIM1 or si-Orai1) induced a dramatic decrease in the SOCE's amplitude but not its abolition, indicating that only part of it might be due to the Orai1/STIM1 complex. The fact that we dramatically abolished by si-RNA STIM-1 definitely demonstrated that DC maturation and maturation marker expression are under the control a CCE involving mainly STIM-1 and Orai-1 as no changes were observed on TRPC expressions [26].

To build a functional channel, tetramers of the Orai1 protein and STIM1 Ca^{2+} sensor must interact to allow Ca^{2+} entry into the cytosol [27]. Indeed, these two proteins appeared to be co-localized in DC stimulated by the maturating agents used. When TNF-α was applied, the maturation markers were lesser expressed than in the one induced with LPS or TG, indicating that the coupling between these two proteins has demonstrated lower intensity. When zymosan is used, these proteins do not seemed not to be as clearly co-localized. The Orai1 labeling on zymosan-treated DC only showed structures as puncta. Indeed, zymosan is taken up in mouse and human DC forming phagocytosis vesicles following membrane modeling [28,29]. Taken together the results obtained with SOCE profiles, siRNA assays and confocal microscopy, the TLR2-agonist zymosan may induce DC maturation using a different molecular calcium pathway than the TLR4-agonist LPS and TNF-α, which remains to be determined. It is therefore possible that different ion channels could be involved in affecting specific SOCE profiles as TRPC, Orai1 and STIM1 that can form heteromeric complexes [27,30] or other members of the TRPC or ORAI families such as Orai3 in cancer cells [31].

Signaling through either TLR2 or TLR4 induces the maturation of DCi and modulates the expression of cytokines, i.e., induces IL-12, IL-10 and IFN-γ synthesis. Depending on the maturating agents used on DC, cytokine secretions appeared differently sensitive to SOCE inhibitors. Because of the DC purification method, we cannot completely spread out the possible effect of contaminating T lymphocytes. Nevertheless, the secretion of IL-12 was totally abolished by 2-APB treatment in LPS-treated DC. We could hypothesize that IL-12 secretion is related to the Ca^{2+} amplitude observed in SOCE. The IFN-γ secretion was less inhibited in zymosan-matured DC in comparison to LPS and TNF-α condition. This secretion might be more related to high and constant cytosolic Ca^{2+} level as demonstrated the continuous part of SOCE profile. We could hypothesize that according to intracellular Ca^{2+} concentration and the kinetics, various pathways might be induced leading to the cytokine secretions. Both TLR and GPCR receptor signaling have substantive roles in the regulation of DC function. Indeed, in mouse and human models, activation of TLR-4, a GPCR, provokes the recruitment of G proteins and their regulators (RGS: regulator of G protein signaling) [32]. Moreover, the cytokine production (IL-12, essentially) is regulated by GPCRs in human monocytic lineage

[33]. The IFN-γ secretion is mainly dependent on calcineurin and NFAT pathways [34]. Another Ca^{2+}-dependent pathway such as the Calmodulin-kinase pathway could be involved in cytokine secretion in DC [35,36]. This pathway is involved in zymosan-activated murine macrophage for IL-10 secretion [37].

Beyond its effect on the early SOCE in DC maturation, the Orai1/STIM1 complex might have an impact on the whole immune physiology through ionic homeostasis in DC as well as in T cells [6]. The clinical phenotype associated with STIM-1 deficiency in patients is very similar to that of Orai1 deficiency, suggesting that both genes act in the same pathway and are critical for SOCE in the same tissues [17]. Additionally, deregulated ion homeostasis has been associated with pathophysiological processes in inflammation and autoimmune diseases [38,39,40,41]. In fact, the Orai1/STIM1 complex because of its role in DC physiology could be a good target candidate for ion channels inhibitors in immuno-modulatory treatments.

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Author Contributions

Conceived and designed the experiments: RF DC JF AD FVR JYLG. Performed the experiments: RF DC AD JF. Analyzed the data: JYLG FVR RF DC JF. Contributed reagents/materials/analysis tools: YL. Wrote the paper: RF FVR JYLG.

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Résumé :

Le récepteur sigma-1 est une protéine chaperonne active dans des tissus lésés. Le récepteur sigma-1 est principalement exprimé dans le cerveau et joue un rôle neuroprotecteur dans l'ischémie ou les maladies neurodégénératives.

Le récepteur sigma-1 est également exprimé dans des lignées cellulaires cancéreuses et des travaux récents suggèrent sa participation dans la prolifération et l'apoptose. Cependant, son rôle dans la carcinogénèse reste à découvrir.

Les canaux ioniques sont impliqués dans de nombreux processus physiologiques (rythme cardiaque, influx nerveux, …). Ces protéines membranaires émergent actuellement comme une nouvelle famille de cibles thérapeutiques dans les cancers. Au cours de ma thèse, j'ai montré que le récepteur sigma-1 régule l'activité du canal potassique voltage-dépendant hERG et du canal sodique voltage-dépendant Nav1.5 respectivement dans des cellules leucémiques et des cellules issues de cancer du sein. J'ai également montré que le récepteur sigma-1, à travers son action sur l'adressage du canal hERG, augmente l'invasivité des cellules leucémiques en favorisant leur interaction avec le microenvironnement tumoral.

Ces résultats mettent en évidence le rôle du récepteur sigma-1 sur la plasticité électrique des cellules cancéreuses et suggèrent l'intérêt de cette protéine chaperonne comme cible thérapeutique potentielle pour limiter la progression tumorale.